이학박사학위논문

CTCF 단백질이 장기 기억과 대뇌피질 시냅스 가소성 조절에 미치는 영향에 대한 연구

Studies on the role of CCCTC-binding factor (CTCF) in remote memory and cortical synaptic plasticity

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ABSTRACT

Studies on the role of CCCTC-binding factor (CTCF) in remote memory and cortical synaptic plasticity

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The molecular mechanism of long-term memory has been extensively studied in the context of the hippocampus-dependent recent memory examined within several days. However, months-old remote memory maintained in the cortex for long-term has not been investigated much at the molecular level yet. Various epigenetic mechanisms are known to be important for long-term memory, but how the three-dimensional (3D) chromatin architecture and its regulator molecules contribute to neuronal plasticity and memory consolidation are still largely unknown. To assess memory upon the perturbation of the transcription and 3D chromatin structure, I chose the CCCTC-binding factor (CTCF), a seven-zinc finger protein well known for its role as a transcription factor and a chromatin regulator. I generated conditional knockout (cKO) mice, in which CTCF is lost in

excitatory neurons during adulthood. The CTCF cKO mice showed normal recent memory in the contextual fear conditioning and spatial water maze tasks. However, they showed remarkable impairments in remote memory in both tasks. Underlying the remote memory-specific phenotypes, I observed that loss of CTCF disrupts cortical long-term potentiation (LTP), but not hippocampal LTP. Through RNA-sequencing, I observed that CTCF knockdown in cortical neuron culture caused altered expression of hundreds of genes. In the list of differentially expressed genes (DEGs), more number of genes were downregulated than upregulated. Also, through gene ontology (GO) enrichment analysis, I found that many DEGs are highly involved in functions such as cell adhesion, synaptic plasticity, and memory. For further investigation on the function of CTCF in the brain, I generated another cKO mouse line, in which CTCF was specifically deleted in inhibitory neurons. However, due to embryonic lethality, I used CTCF heterozygous (CTCF HT) instead of homozygous KO mice for experiments. In the behavioral experiments, I observed partial impairment of remote memory in the CTCF HT mice, which suggests that CTCF has a similar function of regulating remote memory in excitatory and inhibitory neurons. Moreover, as CTCF cKO mice have many number of genes with an abnormal expression level, I looked at CTCF cKO mice's brain to assess the long-term consequences of CTCF deletion. In the hippocampus and ACC of the aged CTCF cKO, I found strong signs of cell death and reactive gliosis, which are marks of neurodegeneration. Together, these results suggest that remote memory storage in the cortex requires CTCF-mediated transcription and chromatin

regulation, while hippocampus-dependent short-term memory does not. Also, the results from aged CTCF cKO mice suggest that long-term CTCF deletion leads to neurodegeneration in two memory-related brain areas.

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Keyword: CTCF, Transcription, Chromatin remodeling, Systems consolidation, Neurodegeneration

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CHAPTER I

INTRODUCTION

BACKGROUND

The formation of long-term memory in the brain involves dynamic gene regulation through multiple layers of mechanisms. This has been established based on studies focusing on the recent long-term memory, which is typically examined one to several days after learning (Huang et al., 2013; Lee et al., 2007; Tsokas et al., 2016). However, memory is believed to be further processed into remote long-term memory over several weeks after the initial consolidation, during which the major brain region preserving the memory is shifted from the hippocampus to the cortex; this process is called systems consolidation (Frankland et al., 2004; Wang et al., 2006). Some previous studies have shown that epigenetic mechanisms are involved in systems consolidation. For example, cortical DNA methylation and histone acetylation participate in the formation and/or maintenance of remote memory (Miller et al., 2010; Peixoto and Abel, 2013; Yu et al., 2011). However, much of systems consolidation process still remains elusive and the exact molecular underpinning of how remote memory is regulated is yet to be discovered.

In this study, I investigated the roles of CTCF in systems consolidation. Using cKO mice, I first assessed the consequences of loss of CTCF at behavioral, electrophysiological, and molecular levels. In the second part of the study, I investigated roles of CTCF in inhibitory neurons in terms of remote memory. Moreover, I examined the result of long-term loss of CTCF and it contributes to abnormal induction of neurodegeneration. In the

introduction of this study, I start by addressing a key data, from which this study has started from, and explain how ideas around studying CTCF have been developed. Then, I lay out background information on a number of terminologies addressed in each chapter and explain how data from this study supports to explain each terminology or phenomenon. Last but not least, I provide an introduction to behavioral methods used in this study and describe how each test or technique is performed and how data are obtained for specific measures.

CTCF protein

Among several chromatin architecture regulators, CTCF is the only known vertebrate protein to exhibit an insulator activity, which guards genes from inappropriate chromatin interactions either by blocking the enhancers from interacting with gene promoters or by inhibiting the spread of heterochromatin (Gaszner and Felsenfeld, 2006). CTCF recognizes the consensus sequence CCGCGNGGNGGCAG using eleven zinc finger motifs (Kim et al., 2007) and can bind to various sequences through combinations of its zinc fingers (Ohlsson et al., 2001) (Figure 1) to mediate DNA looping by binding to enhancers, gene promoters, and gene bodies (Holwerda and de Laat, 2013). Chromatin immunoprecipitation experiments combined with high-throughput sequencing (ChIP-seq) have mapped CTCF binding activities in diverse tissues, revealing that CTCF can bind to 55,000–65,000 sites on the mammalian genome (Ong and Corces, 2014). Many of the CTCF binding

sites are located near gene loci (Hirayama et al., 2012), and CTCF can also bind to itself (Yusufzai et al., 2004) or to other transcription factors, such as cohesin, to form chromatin loops and regulate transcription (Guo et al., 2012). The consensus binding sequence of CTCF contains CpG and can thus be subject to DNA methylation. CTCF preferentially binds to unmethylated sequences, as shown at the H19-Igf2 locus (Phillips and Corces, 2009). Portions of CTCF binding sites are found in transitions between active and inactive chromatin domains (Kim et al., 2015). This shows that CTCF functions to separate different chromatin states. Previous studies have shown that CTCF has a direct role in transcriptional regulation and that CTCF shares DNA binding sites with its binding partner cohesion (Guo et al., 2012). Cohesin is functionally associated with DNA replication, and CTCF can form a protein complex with cohesion that contains proteins involved in structural maintenance of chromosomes (Monahan et al., 2012). In addition to cohesin, CTCF also has other binding partners, such as FOXA1 and TAF3k, which are transcriptional factors (Holwerda and de Laat, 2013). As CTCF can attract many different transcription factors in a tissue- and genomic context-specific manner, CTCF's function at a given genomic site depends on various factors, like associated proteins and engagement in chromatin loops. At the protooncogene *Myb* locus, CTCF binding occurs in the first intron of the gene. CTCF is also found to bind many sites across the immunoglobulin and T-cell receptor antigen receptor gene loci (Kim et al., 2007). Also, CTCF regulates expression of protocadherin-a cluster (Chen and Maniatis, 2013). Pcdhs are known to be important for recognition and diversification of neurons.

Expression of the pcdh isoforms is reduced upon conditional CTCF KO in post-mitotic neurons (Hirayama et al., 2012), and this suggests that CTCF-mediated long-range interactions control transcription of these genes. Various chromatin interaction analysis with paired-end tag sequencing show genome-wide DNA interactions mediated by a protein of interest. When targeted CTCF, ChIA-PET revealed 1500 intra-chromosomal and 300 inter-chromosomal interactions (Ziebarth et al., 2013). CTCF loops contain active chromatin and inactive chromatin. Enhancers and promoters can also be captured in a chromatin loop. More recent studies have shown that CTCF impacts chromosome topology, organizing genes by creating DNA loops. CTCF regulates 3D configuration of the genome, which is critical for dynamic and accurate gene expression (Splinter et al., 2006).

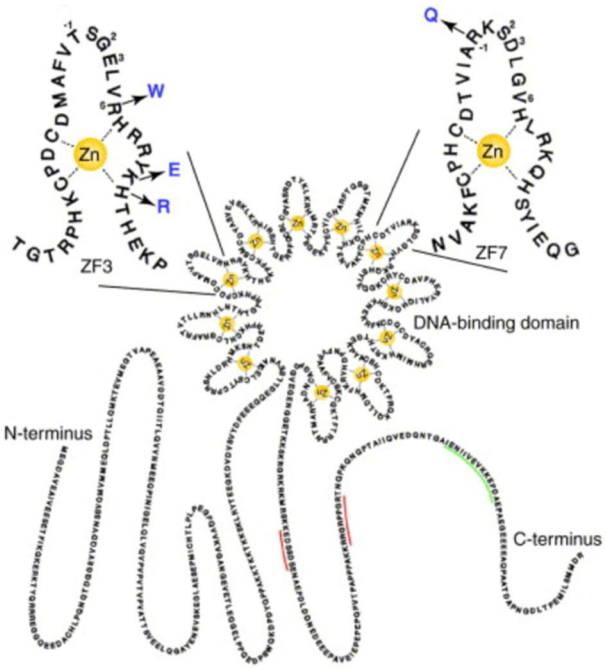


Figure 1. Structural configuration of CTCF

The wild-type human CTCF protein is composed of a total of eleven zinc fingers: ten C_2H_2-class zinc fingers and one C_2HC-class zinc finger. CTCF has functionally significant sites, such as pol II-interacting domain (green). (Adapted from Ohlsson et al., 2001

Previous finding of mLLP interaction with CTCF

In a previous study (Yu et al., 2016) from our lab, the authors discovered that mLLP, a mammalian homolog of *Aplysia* LAPS18-Like Protein (ApLLP), interacts with CTCF and other transcriptional machineries to modulate gene expression involved in neuronal growth. LLP homologs are evolutionarily well conserved proteins, and mLLP is a nuclear/nucleolar protein with cell-permeability conferred by its N- or C- terminal domain (Yu et al., 2016). Yu et al. (Yu et al., 2016) reported a novel mechanism, through which mLLP regulates neuronal development by modulating density of dendritic protrusions and synaptic transmission. After finding that mLLP protein expression is changed upon neuronal activation by KCL treatment in cultured neurons, Yu et al. (Yu et al., 2016) conducted a co-immunoprecipitation assay and found that mLLP interacts with CTCF (Figure 2A). Suggesting that mLLP may play a part in CTCF-mediated gene regulation, Yu et al. (Yu et al., 2016) performed a qRT-PCR analysis, revealing that mRNA level of a subset of CTCF target genes, including amyloid precursor protein (APP) and protocadherin genes (Pcdhs), are downregulated in mLLP knockdown hippocampal neuron cultures (Figure 2B). From these results, Yu et al. (Yu et al., 2016) suggested that mLLP and CTCF may be partners that control gene expression necessary for neural development together.

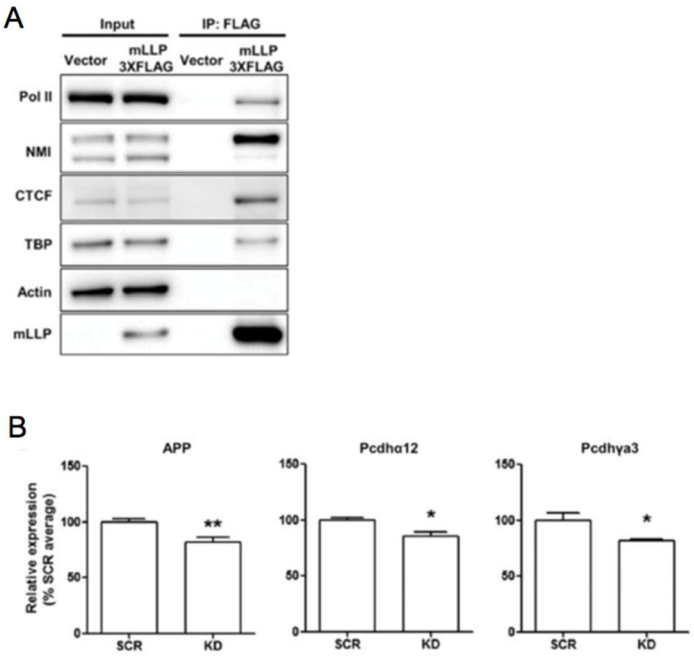


Figure 2. Interaction of mLLP with CTCF

(A) Co-immunoprecipitation assay showing that mLLP interacts with CTCF.

(B) qRT-PCR analysis of CTCF target genes.

(Adapted from Yu et al., 2016)

In 2012, Hirayama et al. (Hirayama et al., 2012) published a paper, showing that CTCF is required for neural development. This was the first report on CTCF's role in the brain. By crossing the floxed CTCF mouse line with *Nex-Cre* mouse line, the authors produced CTCF-cKO mice, which had reduced CTCF expression in post-mitotic cortical and hippocampal neurons. Using their CTCF-cKO mice, Hirayama et al. (Hirayama et al., 2012) showed that the loss of CTCF causes abnormal stochastic expression of *Pcdh* genes and dendritic arborization. Also, CTCF deletion caused changes in the excitatory postsynaptic currents. With results from Yu et al. (Yu et al., 2016) and Hirayama et al. (Hirayama et al., 2012), I wondered if CTCF has a role in the brain other than regulating the neuronal development. I was especially interested in CTCF's role in mature neurons and in long-term regulation of memory because CTCF participates in chromatin remodeling, which may cause chronic changes in the gene expression. I hypothesized that loss of CTCF may cause a disruption in systems consolidation process. Memory consolidation is known to require protein synthesis and epigenetic modifications. Among several different forms of memory, I decided to focus on remote memory in this study because I hypothesized that permanent modifications made on chromatin are likely to induce irreversible changes of the gene expression, which may have a long-term effect on memory consolidation.

Types of memory

Memory can be divided into several types by different measures, but

most often, memories are differentiated by the length of endurance. There are several types of memory, and in this section, I am going to give a short introduction to four types of memory. First, sensory memory holds sensory information until it is transferred to short-term memory. Humans have five senses, including sight, hearing, taste, smell, and touch. Sensory memory is the shortest form of memory, and it only holds impressions of the sensory information after the stimulus is gone (Pasternak and Greenlee, 2005). Secondly, short-term memory holds a small amount of information for a short period of time. Usually, short-term memory can retain information for seconds up to a few days, and information is not open for manipulation (Cowan, 2008). It is important to note that working memory is similar and yet different from short-term memory. Working memory is a type of short-term memory, but it refers to a process of operating the information to carry out a cognitive function. While working memory is not completely distinct from short-term memory, the term "working memory" is used to refer to an ability of our brain to use the information to make a plan and perform a behavior. (Cowan, 2008) Lastly, long-term memory is the longest form of memory that can last a few days to decades. As mentioned above, active protein synthesis is required for long-term memory formation and long-term synaptic plasticity (Roberson and Sweatt, 1999). Identification of key factors involved in the molecular process of long-term memory has been one of the main interests in the field of neuroscience. Well-coordinated transcription processes and gene expression are necessary for establishing appropriate changes to hold the memory for long-term.

Synaptic and systems consolidation

Memory consolidation refers to a set of processes that stabilize a memory trace after acquisition. The term “consolidation” was first created by Muller and Alfons Pilzecker in the late 1800’s (Squire et al., 2015). They proposed that memory takes time to be solidified and new information that comes in before the full solidification can disrupt the previous memory. This introduced an idea of labile nature of a newly formed memory. There are mainly two types of memory consolidation: synaptic consolidation and systems consolidation. Synaptic consolidation occurs within a few hours after learning and requires protein synthesis. On the other hand, systems consolidation occurs over a longer period of time and it can take from weeks to years (Wang et al., 2006). During systems consolidation process, hippocampus-dependent memories become more cortex-dependent. Before going into details, it is worth mentioning “reconsolidation”. It is another type of consolidation, during which previously-consolidated memories become transiently labile through reactivation of the memory. During this process, memories can be edited and be opt for consolidation again. It is a distinct process that allows modification of memories (Dudai, 2004).

Synaptic consolidation is thought start with changes in the membrane potential that activate transduction inside the neuron. The signaling cascades trigger transcription factors to change the expression of various genes(Tonegawa et al., 2015). The active synthesis of synaptic proteins causes remodeling at synapses, which supports the consolidation of a memory trace. Long-term potentiation (LTP) refers to the strengthening of synaptic

transmission and is one of the best understood forms of synaptic plasticity(Volianskis et al., 2015). When LTP occurs, neurotransmitter release, receptor sensitivity, and the number of the receptors at the post-synaptic membrane are increased (Alarcón et al.; Volianskis et al., 2015). As synaptic strengthening is required for memory formation, LTP is thought to be an underlying mechanism of the synaptic consolidation. Compared to systems consolidation, synaptic consolidation is a relatively fast process, taking in place minutes to hours after memory is initially encoded. Synthesis of new proteins is required for the formation of a new memory, and administration of protein synthesis inhibitors, such as Anisomycin, disturbs LTP as well as memory consolidation process (Davis and Squire, 1984).

On the other hand, systems consolidation is a process, during which the hippocampus and neocortex interact with each other and reorganize the stored information (Wang et al., 2006). Through systems consolidation, memories end up in a more permanent form stored in the neocortex. Previous studies on retrograde amnesia have provided early evidence for systems consolidation (Squire et al., 2015). Studies had found that remote memories are less vulnerable to disruption than recent memories. Since then, many researchers have attempted to reveal what brain regions and mechanisms are responsible for the systems consolidation process. More recent studies have started to provide evidence for the neural mechanisms underlying the interaction of hippocampus and neocortex. The concept of systems consolidation is not that memory traces are literally transferred from hippocampus to the neocortex. In fact, information is also encoded in the

neocortical regions at the time of learning. Instead, the idea is that gradual changes strengthen the connectivity among the cortical regions while connectivity to hippocampus is weakened, which establishes a stable cortex-dependent long-term memory (Santini et al., 2004). When memory is initially formed, it is labile in nature (Abel and Lattal, 2001). It is susceptible to environmental stimuli-induced modifications and can be easily reconstructed. Therefore, to sort out various incoming information and store specific information for long-term in a correct form, consolidation processes are necessary. To store life-long memory, a process called epigenetics was found to be necessary on top of transcription (Monsey et al., 2011). Because the timescale of protein turn-over is within hours, more stable and long lasting molecular mechanisms are required. Epigenetic modifications are self-perpetuating in nature and can protect memory traces from molecular changes induced by various stimuli (Woldemichael et al., 2014). Studies on epigenetic mechanisms showed that these biochemical changes help propagate memories over a lifetime (Lipsky, 2013). Epigenetic regulations are active in post-mitotic neurons and reserved across species, from *Aplysia* to rodents. Because epigenetic markers are stable, they allow dynamic experience-dependent regulation of the genome and memory maintenance.

In this study, I focused on the systems consolidation and explored the role of CTCF in consolidating memory over 4 weeks of time. As systems consolidation takes weeks to years, molecular underpinnings of this process are less studied than the synaptic consolidation. Therefore, in this study, I investigated novel roles of CTCF during the transfer of memory to the neo-

cortex and assessed the effect of CTCF deletion on memory maintenance.

Transcriptional regulation

Transcription or protein synthesis is a highly regulated process that involves combined interaction of chromatin and proteins, such as CREB and CBP (Yin and Tully, 1996). Studies have shown that gene expression is organized in process of cascades, starting with the expression of regulatory immediate early genes that first respond to extracellular stimuli (Silva et al., 1998). Transcription is a crucial process underlying long-term memory. As memory traces are transformed into long-term memory, cell state changes are induced. Transcription activators and repressors are both recruited, and modifications are made on chromatin and at specific loci (Alberini, 2009). One example of a transcription factor is the cAMP responsive element binding protein (CREB). CREB is a nuclear protein that regulates transcription of other genes by binding to cAMP responsive element (CRE) DNA sequences. CREB induces the expression of genes like c-fos, BDNF, and tyrosine hydroxylase (Cardinaux et al., 2000). An increase of calcium or cAMP concentration can trigger phosphorylation and activation of CREB (Igaz et al., 2004). Yin et al. (Yin and Tully, 1996) have previously shown that CREB-dependent transcription is specifically required for long-term memory. They also observed increasing the amount of CREB accelerates the memory formation process. Compared to short-term memory, long-term memory requires more intricate processes with synthesis of new proteins to store

information that can be recalled later.

Epigenetics

Epigenetics refers to heritable changes in gene expression that do not involve alteration of DNA sequences. Examples of epigenetic modification includes methylation of DNA or histone (Woldemichael et al., 2014). Epigenetic mechanisms regulate DNA compaction and appropriate gene expression. For massive chromosomes to fit into nucleus and stay in an organized form, every 146 bp section of DNA is coiled around an octamer of histone proteins. A nucleosome is a basic unit of DNA packaging, consisting of eight histone proteins wrapped around by a segment of DNA (Portela and Esteller, 2010). To carry out various cellular processes, the formation of chromatin must actively change. And the switching between open and closed form of chromatin and the accompanied assembly of transcriptional machinery at gene promoters are mediated by epigenetic modifications (Kouzarides, 2007). Transient epigenetic markers observed in hippocampus suggest initial but temporary involvement of hippocampus in this process (Levenson et al., 2004). In contrast, older memories depend on the cortex for maintenance, and they heavily rely on altered gene expression and epigenetic modifications in the cortex. These changes enable long-term modifications that assist the memory storage. For example, DNA methylation involves an addition of a methyl group to cytosine of DNA. It is known of lead to transcriptional repression of a number of genes (Day and Sweatt, 2010). Day et al. (Day and Sweatt, 2010) have observed that blocking DNA methylation

by applying DNA methyl transferase (DNMT) inhibitors about 30 days after training disenable the memory recall. This indicates that stable cortical DNA methylation and specific gene repression support the maintenance of memory over time. Other types of epigenetic mechanism include histone methylation, acetylation, and phosphorylation. Histone acetylation is one of the most studied epigenetic mechanisms, and it involves a replacement of a hydrogen with an acetyl group to lysine tail of the histone (Federman et al., 2009). It is processed by enzymes that have histone acetyltransferase (HAT) activity. Histone acetylation increases DNA accessibility for transcription factors to bind to gene loci. Histone acetylation is highly reversible, and histones can be de-acetylated by histone deacetylases (HDACs) (de Ruijter et al., 2003). P300 is a transcriptional activator protein that interacts with CBP to regulate other transcription factors (Korzus et al., 2004). P300 and CBP have histone acetyltransferase (HAT) activities, and they have been shown to be critical for long-term memory formation. Mutant mice expressing p300 mutant exhibit impaired long-term recognition and contextual fear memory (Oliveira et al., 2007). This study suggests that P300's acetyltransferase enzymatic activity is necessary for long-term memory.

Chromatin remodeling

Recently, three-dimentional (3D) chromatin architecture has been receiving increasing attention in the research on epigenetic mechanisms (Bonev and Cavalli, 2016). Chromatin remodeling allows chromatin interaction within the topologically associating domains (TADs) and long-

range interaction of enhancer and promoter through DNA looping. This provides important structural bases for gene regulation, thereby contributing to the cell-type specific gene expression (Bouwman and de Laat, 2015). In both mouse models and human patients, the disruption of 3D chromatin architecture has been shown to be associated with neuropsychiatric diseases such as Alzheimer's disease, post-traumatic stress disorder, and schizophrenia (Medrano-Fernández and Barco, 2016). However, the role of 3D chromatin remodeling in learning and memory has only recently been acknowledged (Bharadwaj et al., 2014; Ito et al., 2014), and, in particular, its contribution to remote memory has not been addressed to date. Chromatin remodeling affects the regulation of gene expression by changing the relationship between nucleosomes and DNA (Jin et al., 2005). Histone acetylation removes positive charge, which reduces the level of interaction between formerly positively charged histone and the negatively charged phosphate groups of the DNA (Graff and Tsai, 2013). This change in charge causes a relaxation of DNA from the nucleosome and acetylated DNA has higher levels of gene expression.

Description of behavioral tests

In this study, I used different mouse behavior assays. Mouse behavioral testing is widely used in the field of neuroscience. A detailed description of behavioral test schemes are can be easily found (Crawley, 2008; Silverman et al., 2010), and labs around the world use various behavioral tests to measure the changes of behavioral output after genetic or pharmacological

manipulations in the animal. In this study, two behavioral tests were mainly used: Morris water maze (MWM) and contextual fear conditioning (CFC).

MWM tests spatial memory in an open swimming arena (Figure 3A). It has been shown that MWM is highly correlated with hippocampal synaptic plasticity and NMDAR function (Vorhees and Williams, 2006). In this test, mice are trained to swim and learn to locate a platform that is hidden underwater. Often, mice are encouraged to use spatial cues around the swimming arena while locating the platform. After about a week-long training, mice are put in a probe test, during which the platform is removed, and the mice are tested for the reference memory for the probe's location. A number of measures can be recorded during the probe test, such as the number of platform crossings and the time spent in the quadrant that platform was previously located.

Secondly, CFC tests the ability of mice to learn an association between environment or context and aversive memory (Shoji et al., 2014) (Figure 3B). Usually, aversive memory is fear memory. During a conditioning phase, mice are placed in a chamber and is given an electric foot shock during. After a delay of time, mice are placed back in the same chamber without any electric shock. Mice's freezing or immobile behavior is measured as an index of associative fear memory, assessing whether mice remember the aversive context in the absence of actual stimuli (Shoji et al., 2014).

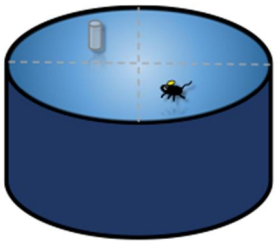
On the other hand, open field test and elevated zero maze are simpler tests that measure anxiety and spontaneous activity (Figure 3C). Open field

test is used to assay the locomotor activity level and exploratory behavior. Mice are put in a box-like arena, in which mice are free to explore. The open field is mainly divided into two parts: center area and periphery area. As mice have natural instinct of staying in the periphery area close to the walls, the motion detector above the arena measure mice's locomotion and the experimenter analyze how much time the mice spent in each area as well as the distance moved. The time spent in the center or periphery area can be used as a measure the anxiety level. For example, mice with relatively higher anxiety level would spend more time in the periphery area than the control mice.

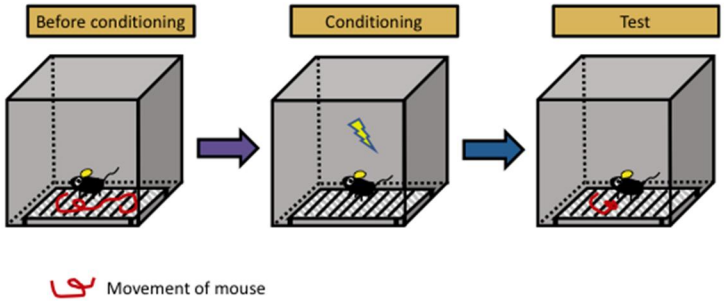
Elevated zero maze is used to assess anxiety-like behavior (Figure 3D). In this task, an elevated zero- or circle-shaped apparatus consisting of two open and two enclosed areas is used. The enclosed areas are made by two opaque walls that create dark, secure area in between. This test uses mice's nature to explore the novel environment, while also innately fearing the height and the open area. Anxiogenic mice tend to spend more time in the enclosed area than the open area. The time spent in enclosed versus open arms is used as a measure of the anxiety level of the animal.

While requiring precise handling of mice with accurate protocols, the use of the two behavioral tests added robust and reliable results to this study.

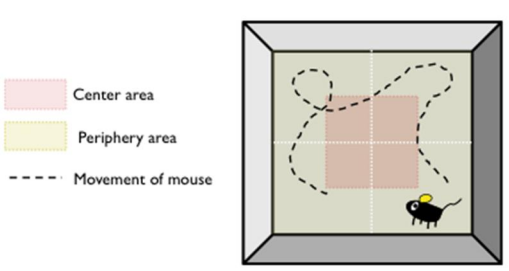
A



B


Before conditioning
Conditioning
Test
Movement of mouse

C


Center area
Periphery area
Movement of mouse

D

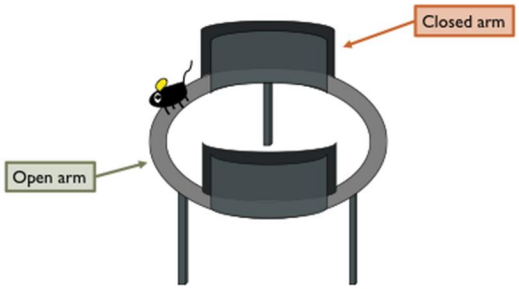

Closed arm
Open arm

Figure 3. Behavioral tasks used in this study

(A) Schematic image of Morris water maze (MWM).

(B) Schematic image of contextual fear conditioning (CFC).

(C) Schematic image of open field test (OFT).

(D) Schematic image of elevated zero maze (EZM).

PURPOSE OF THIS STUDY

As previous studies on epigenetic mechanisms have shown that memory is affected by modifications made on the chromatin by various proteins, I became interested in the relation between transcription, 3D chromatin structural remodeling, and memory. Building on the previous results on CTCF's role during development (Hirayama et al., 2012; Yu et al., 2016), I used the *Cre/lox* system to delete CTCF in specific type of neurons in mice and examined the function of CTCF in relation to remote memory. CTCF is well-known for its role as a transcription factor and a chromatin remodeler, but little is known about its role in the brain. In this thesis, I address the function of CTCF in mature excitatory neurons at behavioral and physiological levels, using several behavioral test schemes and extracellular field recording techniques. Moreover, I examine the function of CTCF in inhibitory neurons in relation to remote memory and how long-term loss of CTCF induces signs of neurodegeneration.

In chapter II, I begin by addressing the first goal of this study, which is to generate a CTCF deficient mouse model with sufficiently low level of CTCF expression in excitatory neurons. Then, I identify memory deficits by using several different behavioral tasks. I also present extracellular field recording data of hippocampal and ACC slices and identify physiological phenotypes of the CTCF cKO mice. Lastly, I discuss the results from RNA-seq of CTCF KD cortical neuron culture, showing molecular markers underlying the behavioral and electrophysiological phenotypes (Figure 4).

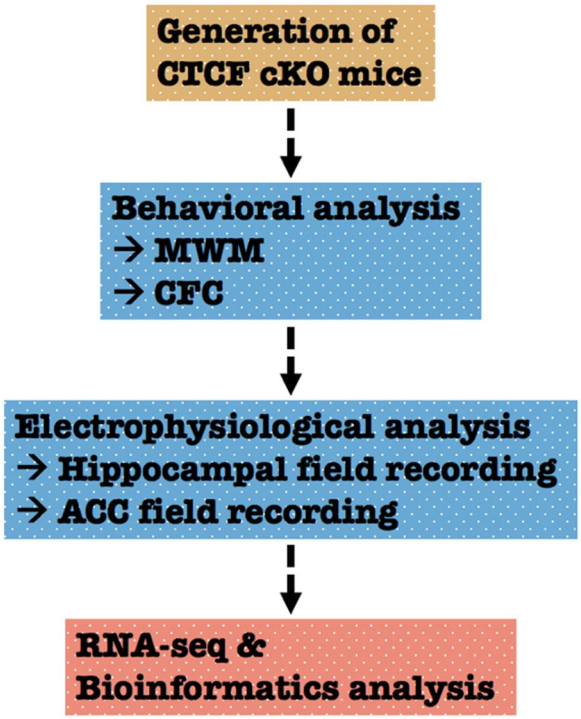


Figure 4. Workflow of chapter II

In chapter III, I examine behavioral and electrophysiological phenotypes of CTCF HT mice and show roles of CTCF protein in inhibitory neurons (Figure 5A). Moreover, using the CTCF cKO mice from chapter II, I discuss how long-term CTCF deficiency leads to signs of early neurodegeneration, including cell death and reactive gliosis (Figure 5B).

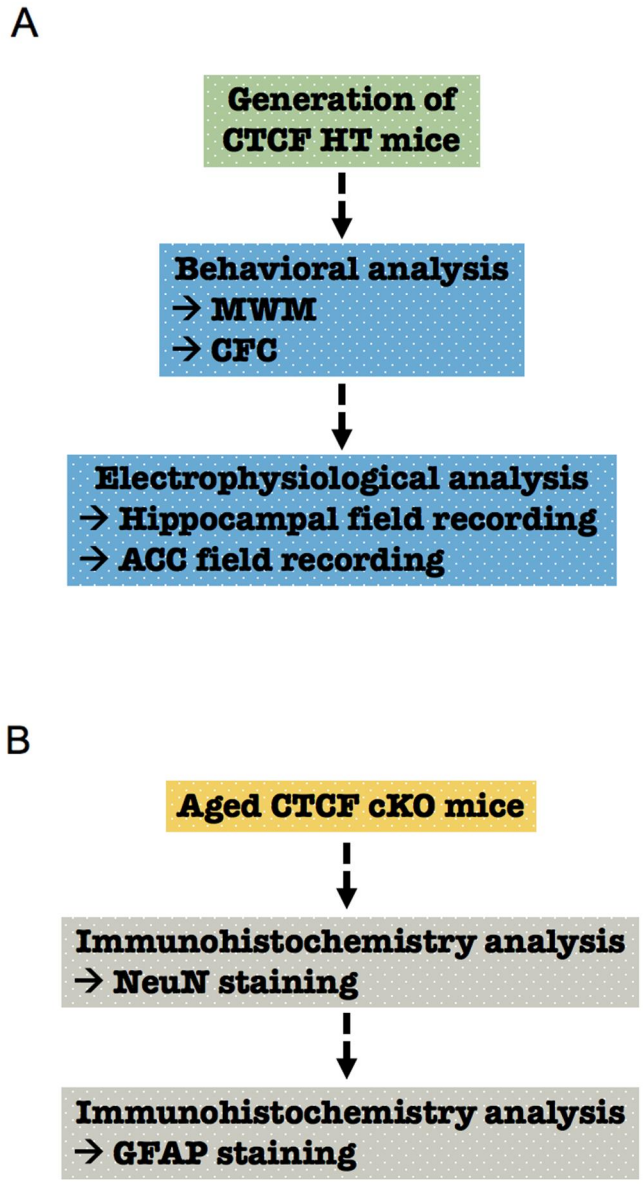


Figure 5. Workflow of chapter III

CHAPTER II

CTCF-mediated chromatin remodeling in forebrain excitatory neurons is necessary for remote memory and cortical synaptic plasticity

INTRODUCTION

As a highly conserved protein, CTCF is also called the "master weaver of the genome" (Phillips and Corces, 2009) due to its multivalent characteristic. CTCF is implicated in widespread cellular regulatory functions, such as transcriptional activation/inhibition, insulation, and imprinting (Phillips and Corces, 2009). For example, as an insulator, CTCF can stop repressive effect of heterochromatin or protect specific genes from enhancer function (Herold et al., 2012). There have been genome-wide studies that provide evidence of CTCF-mediated intra- and inter-chromosomal contacts at genomic loci (Ong and Corces, 2014). Studies suggest that CTCF has a role in the global organization of the chromatin architecture (Splinter et al., 2006). Chromatin is dynamically regulated, transiently forming euchromatin or heterochromatin. Euchromatin is a lightly packed and open form of gene-rich chromatin, which is often under active transcription. On the other hand, heterochromatin is a condensed and closed form of chromatin that often leads to transcriptional repression. CTCF was initially discovered in 1990, as a negative regulator of the chicken *c-myc* gene (Lobanenkov et al., 1990) and soon was proposed that it "customizes its conformation to engage different zinc fingers" to make contact with DNA or other proteins (Ohlsson et al., 2001). Since then, CTCF's role on genome folding and gene expression has been extensively explored at individual locus, such as chicken B-globin and H19-IGF2 (Fedoriw et al., 2004). Also, using a chromosome conformation capture assay and a high-throughput sequencing technique, about 15 million

CTCF-binding sites across 10 species have been found (Ziebarth et al., 2013).

To assess CTCF's role in the adult mouse brain, I chose a breeding strategy, through which I can avoid CTCF deletion during development and specifically cause the loss of protein during adulthood. In the previous study on CTCF by Hirayama et al. (Hirayama et al., 2012), the CTCF-cKO mice died within a month after birth. Thus, this CTCF-cKO mouse model was not applicable to this study. Instead of the *Nex-Cre* line, I used *CaMKIIa-Cre* line, which starts to express Cre recombinase around 4 weeks of age (Tsien et al., 1996). The *Cre/lox* system is a powerful tool that provides a genetic switch of a target gene expression in a region- or cell-type specific manner. Since it also provides time point-specific gene regulation, it is widely used in the field of neurobiology for studying function of specific genes (Carter and Shieh, 2015; Kriegebaum et al., 2010). The basic strategy of the *Cre/lox* system relies on a site-specific DNA recombination by Cre, a 38 kDa protein originally derived from bacteriophage P1 (Tronche et al., 2002). Cre recognizes a 34-bp DNA sequence called *loxP* and catalyzes a reciprocal DNA recombination between the two *loxP* sites (Tronche et al., 2002). The recombination causes an excision of the target allele, resulting in gene deletion.

In this chapter, I used several bioinformatics tools to analyze the data from RNA sequencing (RNA-seq). RNA-seq is one of the high-throughput sequencing methods that uses the next-generation sequencing (NGS) to reveal the presence and quantity of RNA in a biological sample at a given moment in time. While there are several different NGS platforms, they commonly perform sequencing of millions of DNA sequences in parallel, revolutionarily

reducing the time while increasing the accuracy compared to previous sequencing methods (Behjati and Tarpey, 2013). One of the bioinformatics tools I used in this chapter is the ingenuity pathway analysis (IPA). IPA is a powerful tool that identifies target biomarkers within the context of a biological system (Kramer et al., 2014). Relying on its comprehensive database and algorithms, IPA helps scientists discover significant pathways and networks with causal relationships from their data. Also, I used gene ontology (GO) term enrichment, which is a technique that classifies genes based on the gene ontology system (Chen et al., 2009). Genes are categorized by their functional characteristics and assigned to a set of predefined terms. GO term analysis produces a functional profile of gene sets that help better understand the data.

In the present study, I used the *Cre/lox* system to generate CTCF cKO mice and assessed the role of CTCF in forebrain excitatory neurons. Using behavioral experiments, I examined the CTCF cKO mice in CFC and MWM tests. For electrophysiological experiments, I used the conventional field recording system as well as the MED64 system to measure the field excitatory postsynaptic potentials (fEPSPs) in two different brain regions: Hippocampus and ACC. As a result, I found that CTCF cKO mice have disrupted remote memory and cortical synaptic plasticity. Underlying these phenotypes, I found alteration of gene expression in the CTCF cKO. These results support my original hypothesis that CTCF regulates remote memory through transcriptional and chromatin remodeling regulation.

EXPERIMENTAL PROCEDURES

Animals

CTCF cKO mice were generated by crossing CTCFfl/+;CaMKIIαCre/+ with CTCFfl/+;CaMKIIα+/+. Littermates that did not carry the Cre transgene or the floxed CTCF were used as controls. 12- to 15-week old adult male and female mice were used for the molecular, behavioral, and electrophysiological experiments. All animals were housed under a 12-h light/dark cycle with food and water provided *ad libitum*. The Animal Care and Use Committee of Seoul National University approved the animal protocols.

Western Blot

Mouse brain tissues were homogenized in RIPA buffer with the protease inhibitor cocktail (Roche). The protein concentrations were measured using the BCA reagents (Thermo), and the equal amounts of proteins across animals were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The CTCF protein was detected using the antibody (Abcam), and their levels were normalized to the GAPDH (Ambion) measured in the same lanes. The chemiluminescence of ECL substrate catalyzed by HRP-conjugated to the secondary antibody was detected and measured by ChemiDoc system (BMS).

Behavioral Tests

Male mice at 12–15 weeks of age were used for the behavioral analyses in this study. The behavioral experiments were performed essentially following our previous study (Kim et al., 2016). For the Morris water maze, mice were handled daily for 3 min over a week before training. The water maze was a gray cylinder-shaped tank (140 cm diameter, 100 cm height) placed in a room with multiple spatial cues and dim light. Water mixed with white paint (19–21 °C) was filled up to 1 cm above the escape platform (10 cm diameter). Mice were trained for four trials per day with 1 min intertrial intervals. Mice were placed into the different edge points of the maze in each four trials, facing the inner wall of the tank, and tracked using the Ethovision software (Noldus). The order of releasing point was changed daily. When mice reached the platform within 60 s, they were removed from the maze and returned to the transport cage. When they failed, they were guided to or placed on the platform and were subsequently removed from the maze. After 5 days of training, a probe test was performed after removing the platform. Mice were placed at the center of the maze and tracked for 1 min. One more session of training was performed after the probe test. To check the remote memory, the probe test was performed 4 weeks after the last training. For the contextual fear conditioning, mice were placed into the Coulbourne fear conditioning chamber. After 148 s, they received foot shocks (2 s, 0.75 mA) twice with 30 s interval. After 30 s, they were returned to the home cage. Contextual fear memory was tested by placing the mice again in the conditioning chamber and measuring the freezing levels (immobility) for 4 min using Freeze Frame

software (Coulbourne).

Electrophysiology

Hippocampal field excitatory postsynaptic potential (fEPSP) recording

Field excitatory postsynaptic potential (fEPSP) recordings were performed as described previously (Park et al., 2014). After anesthetization with isoflurane, mice were decapitated and their brains were removed. Transverse hippocampal slices were sectioned 400 μm thick using a vibratome (Leica, Hesse, Germany). The slices were retained at 32 °C for 30 min during the recovery period and then incubated at 28 °C until the experiment. All incubation chambers were submerge-fashioned and the artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 2.5 mM KCl, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 2.6 mM $CaCl_2$, 1.3 mM $MgSO_4$) was oxygenated with 95% O_2 and 5% CO_2 and perfused at 1 ml/min throughout the experiment. fEPSPs were recorded from the Schaffer collaterals (SC) of CA1. Stimuli were given every 30 sec using concentric bipolar electrodes (MCE-100; Kopf Instruments) and the responses were recorded using a glass pipette electrode filled with ACSF (1 MΩ). Field potentials were amplified, low-pass filtered (GeneClamp 500; Axon Instruments), and then digitized (NI PCI-6221; National Instruments) for measurement. Data were monitored, analyzed online, and reanalyzed offline using the WinLTP program (WinLTP Ltd., winltp.com, The University of Bristol, UK). For the LTP and LTD experiments, stimulation was provided at the intensity that produces roughly 40% of the slice's maximum slope. Two

responses elicited per minute were averaged and expressed relative to an average of the 20-min baseline responses. Theta burst stimulation (TBS) protocols were used to induce E-LTP and L-LTP (five pulses of 100 Hz repeated five times at 5 Hz; 10 s inter-train interval used for E-LTP; 10 min inter-train interval for L-LTP). The fEPSP response average of the last 5 and 10 min of the E-LTP and L-LTP experiments were used to compare the level of synaptic plasticity between the groups.

ACC field potential recording using multi-electrode array

For ACC multi-electrode array experiments, three to four 300-μm thick coronal brain slices after the corpus callosum connection were sectioned using a vibratome. The slices were incubated in a submerged chamber at room temperature until the experiment. ACSF (124 mM NaCl, 2.5 mM KCl, 1 mM NaH2PO4, 25 mM NaHCO3, 10 mM glucose, 2.5 mM CaCl2, 1 mM MgSO4) was oxygenated with 95% O_2 and 5% CO_2 and perfused at 2–3 ml/min throughout the experiment. The MED64 system (Parasonic, Osaka, Japan) was used as previously described (Kang et al., 2012). A slice was placed on the MED64 probe (MED-P515A, 8 × 8 array, interpolar distance 150 μm, Parasonic) and perfused with ACSF at 28–30 °C. The electrical stimulation (1–20 μm, 0.2 ms) was given to a channel in the deep layer region. MED64 Mobius was used for data acquisition and analysis. One pulse was given per minute and the data were averaged every 4 min. The percentages of the last 4 min (E-LTP) and 8 min (L-LTP) fEPSP slopes were normalized to the averaged value of the 20-min baseline.

Quantitative RT-PCR

Total RNA from tissues or cell culture was extracted using TRIZOL or RNAiso and reverse-transcribed using Superscript III following the manufacturers' instructions. Using the cDNA as templates, quantitative PCR was performed using SyBR premix ExTaqII (Takara) on ABI7300. The 2^-dCt method was used to measure the relative mRNA level of each gene of interest.

Histology, immunohistochemistry, and imaging

Mice were anesthetized by isoflurane and transcardially perfused with 4% paraformaldehyde (PFA) in PBS for tissue fixation. Brains were kept in the PFA solution at 4°C overnight for further fixation. Then, brains were moved to 30% PBS-based sucrose solution for 2 days for dehydration. After the dehydration is completed, brains were frozen and cut into coronal slices using cryostat (Leica Ltd., Germany). Hippocampus was cut into 40μm thick slices, while ACC was cut into 30μm thick slices. Brain slices were then washed with PBS, blocked with blocking solution, and incubated with primary and secondary antibodies consecutively. After the antibody application, the slices were mounted on slide glasses with 50% PBS-based glycerol solution for imaging.

Fluorescence images were acquired using a confocal microscope and analyzed with ImageJ program.

RNA Extraction and Sequencing

Total RNA from cultured cortical neurons was extracted and the integrity and quality of the extracted RNA were assessed by BioAnalyzer. The standard illumina protocol was used to make sequencing libraries for RNA-Seq. Using gel electrophoresis, ~300 bp fragments were isolated and amplified by PCR and sequenced using the illumina HiSeq 2000 in the paired-end sequencing mode (2x101 bp).

RNA-Seq Read Processing and Differential Gene Expression Test

RSEM (RNA-Seq by Expectation Maximization, v1.2.28) (Li and Dewey, 2011) was used to align the raw sequencing reads to the mm10 mouse genome. Only uniquely and properly mapped read pairs were used for further analysis. To assess gene expression levels, the transcript per million (TPM) measure was calculated (Wagner et al., 2012) using the read counts of each gene annotated in Ensembl release 82 (Yates et al., 2015). The EdgeR package (Robinson et al., 2010) in R was used to identify the differentially expressed genes between the CTCF KD (shCTCF) and WT (shLacZ) samples. Differentially expressed genes were defined as those with changes of at least 1.2-fold between samples at a false discovery rate (FDR) of 5%. GO term enrichment analysis on the differentially expressed genes was executed through ToppGene Suite (https://toppgene.cchmc.org/) (Chen et al., 2009) and the network analysis was performed using QIAGEN's Ingenuity Pathway Analysis software (IPA®, QIAGEN Redwood City,

www.qiagen.com/ingenuity).

Experimental Design and Statistical Analysis

All experiments were performed in a blind fashion. I used the minimum number of mice that can produce statistical validity. I used 8-16 mice for behavioral tests and 6-10 for electrophysiology. There were no sex-related differences observed in the CTCF cKO mice, so both male and female mice were used for the experiments.

Data were represented by the mean ± standard error of the mean (SEM). For each variable, comparison of two groups were made using student's t-test. Two-way ANOVA and *post-hoc* Bonferroni test were used for further comparisons. The statistical significance level was set at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

Generation of CTCF cKO mice with CTCF deletion in forebrain excitatory neurons

To evaluate the roles of 3D chromatin architecture in learning and memory, I first induced the disruption of 3D chromatin architecture regulation by genetically deleting CTCF. Previously, Hirayama et al. (Hirayama et al., 2012) demonstrated that the deletion of CTCF in cortical and hippocampal neurons during postnatal development causes abnormal neuronal development, obvious growth retardation, and early lethality within 4 weeks after birth. Because the early lethality prevents examining mice in behavioral and electrophysiological tests, I generated CTCF conditional knockout (cKO) mice by crossing the floxed CTCF line with *CaMKIIa-Cre* line, which expresses Cre recombinase in the forebrain excitatory neurons starting from 4–5 weeks of age. This enabled us to circumvent the lethal effect of postnatal CTCF deletion and the CTCF cKO mice were viable at least until ~8 months of age with no apparent health abnormalities. I confirmed that CTCF protein is sufficiently deleted in the cortical and hippocampal excitatory neurons of the 12-week-old mice using immunohistochemistry and western blotting in the anterior cingulate cortex (ACC) and hippocampus (Figures 6A–C). The residual protein in the CTCF cKO mice shown in Figure 1C is probably due to the intact protein expression in inhibitory neurons and glial cells.

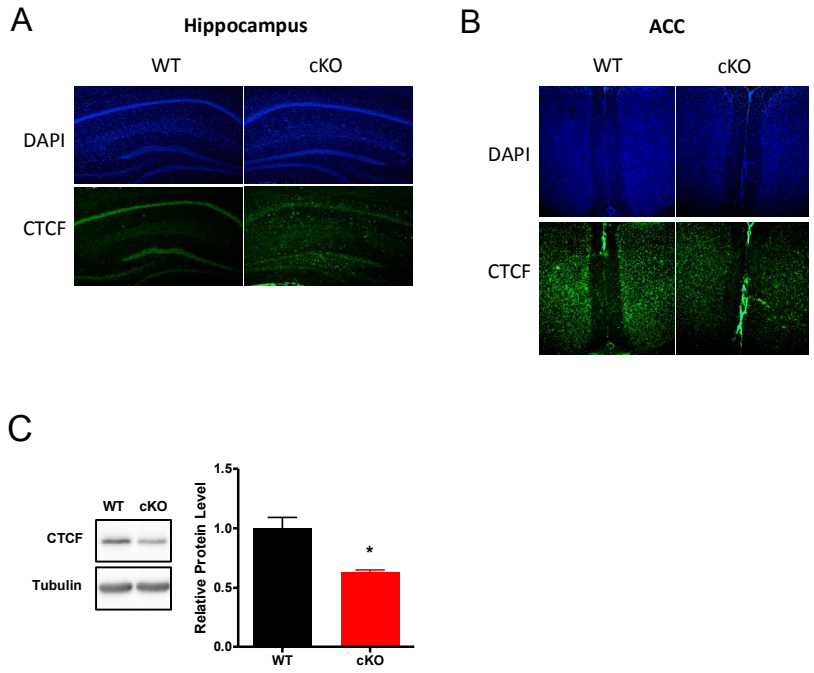


Figure 6. Confirmation of reduced expression of CTCF in CTCF cKO mice

(A) Immunohistochemistry analysis showed that CTCF cKO mice have reduced CTCF protein expression in the hippocampal CA1 region (Blue: DAPI, Green: CTCF, Left: WT, Right: CTCF cKO).

(B) Immunohistochemistry analysis showed that CTCF cKO mice have reduced CTCF protein expression in the ACC (Blue: DAPI, Green: CTCF, Left: WT, Right: CTCF cKO).

(C) Western blot analysis also confirmed that CTCF protein level is reduced in CTCF cKO mice (WT, n = 5; cKO, n = 3).

CTCF-mediated chromatin remodeling is necessary for remote memory

To examine whether the CTCF-mediated chromatin remodeling is needed for the formation of remote memory, I trained CTCF cKO mice on hippocampus-dependent memory tasks. Subsequently, I tested the mice at two different time points: 1 day or 4 weeks after the behavioral training. I hypothesized that if the CTCF-mediated chromatin regulation is necessary for remote memory, the mice would specifically exhibit impairments in the tests performed 4 weeks after the training. When the CTCF cKO mice were first tested 1 day after the training in the contextual fear conditioning (CFC), they displayed comparable freezing levels to the wildtype (WT) mice (Figure 8A). Similarly, in the Morris water maze (MWM), the CTCF cKO mice exhibited normal spatial reference memory during the probe test (Figures 8C, D). The CTCF cKO mice did show a delay in locating the platform during the training (Figure 8B), but this is possibly due to an impaired swimming speed (Figure 8G, H). After 4 weeks, I re-tested the same mice and found that the CTCF cKO mice have a significantly impaired remote memory. In the CFC, the CTCF cKO mice exhibited a lower level of freezing than the WT controls (Figure 8A). Similarly, the CTCF cKO mice displayed a significantly lower count of platform crossing during the probe test (Figures 8E, F). These results confirmed my hypothesis that the CTCF-mediated chromatin regulation in the adult forebrain is not necessary for the formation of recent memory, but is indispensable for the formation of remote memory. To make sure that differences in the behavioral tests are not due to the altered anxiety level, I

tested the CTCF cKO mice in open field test (OFT) and elevated zero maze (EZM). While I found that CTCF cKO mice exhibit significantly more hind-limb clasping behavior, which is a general measure of indication for a cognitive deficit (Guyenet et al., 2010) (Figure 7A), I found that there is no difference in the level of anxiety or locomotion between the CTCF cKO and WT mice (Figures 7B-D).

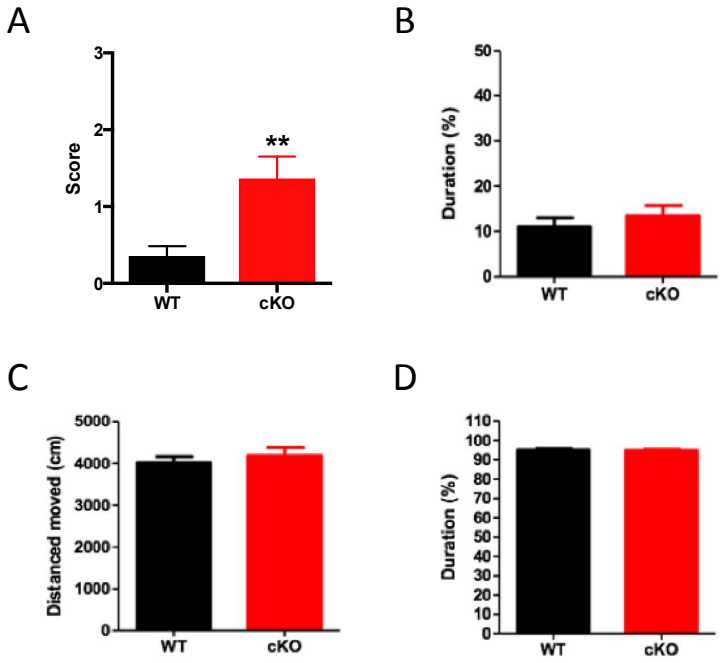


Figure 7. CTCF cKO mice show hind-limb clasping with normal level of anxiety-like behaviors and spontaneous locomotion

(A) CTCF cKO mice exhibited significantly higher hind-limb clasping score (WT, n=16; 0.3594 ± 0.1228 %; cKO, n=13; 1.365 ± 0.2853 %; unpaired *t*-test; $p = 0.0050$).

(B) Open arm duration of CTCF cKO mice in the EZM (WT, n=14; 11.31 ± 2.594 %; cKO, n=12; 10.02 ± 1.635 %; unpaired *t*-test; $p = 0.6902$).

(C) Total distance moved of CTCF cKO mice in the OFT (WT, n=14; 3797 ± 189.9 cm; cKO, n=12; 3983 ± 198.4 cm; unpaired *t*-test; $p = 0.5072$).

(D) Duration in the periphery of CTCF cKO mice in the OFT (WT, n=14; 95.91 ± 0.6961 %; cKO, n=12; 95.12 ± 0.5669 %; unpaired *t*-test; $p = 0.3975$).

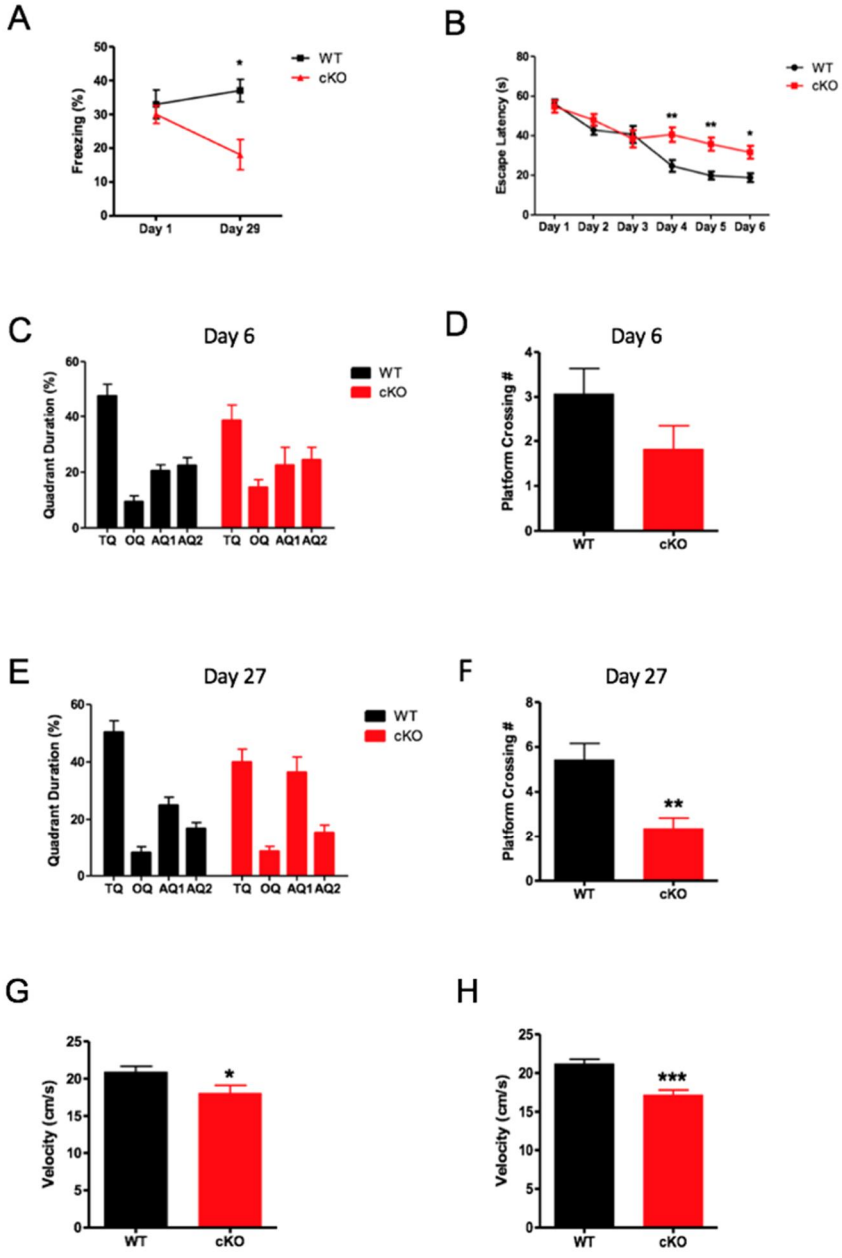

A
Freezing (%)
WT
cKO
Day 1
Day 29
B
Escape Latency (s)
WT
cKO
Day 1 Day 2 Day 3 Day 4 Day 5 Day 6
C
Day 6
Quadrant Duration (%)
WT
cKO
TQ OQ AQ1 AQ2 TQ OQ AQ1 AQ2
D
Day 6
Platform Crossing #
WT
cKO
E
Day 27
Quadrant Duration (%)
WT
cKO
TQ OQ AQ1 AQ2 TQ OQ AQ1 AQ2
F
Day 27
Platform Crossing #
WT
cKO
G
Velocity (cm/s)
WT
cKO
H
Velocity (cm/s)
WT
cKO

Figure 8. CTCF cKO mice display impaired remote memory in two different behavioral tasks

(A) In the CFC test, CTCF cKO mice showed intact recent fear memory but impaired remote fear memory (WT, n = 8; cKO, n = 9; two-way repeated measures ANOVA; effect of interaction, $p = 0.0068$; Bonferroni *post-hoc* test for Day 29, $p < 0.01$).

(B) During the training phase of the MWM, CTCF cKO mice showed significantly longer escape latency on Days 4–6 (WT, n = 14; cKO, n = 11; two-way repeated measures ANOVA, effect of interaction, $p = 0.0009$; Bonferroni *post-hoc* tests, Day 4, $p < 0.01$, Day 5, $p < 0.01$, Day 6, $p < 0.05$).

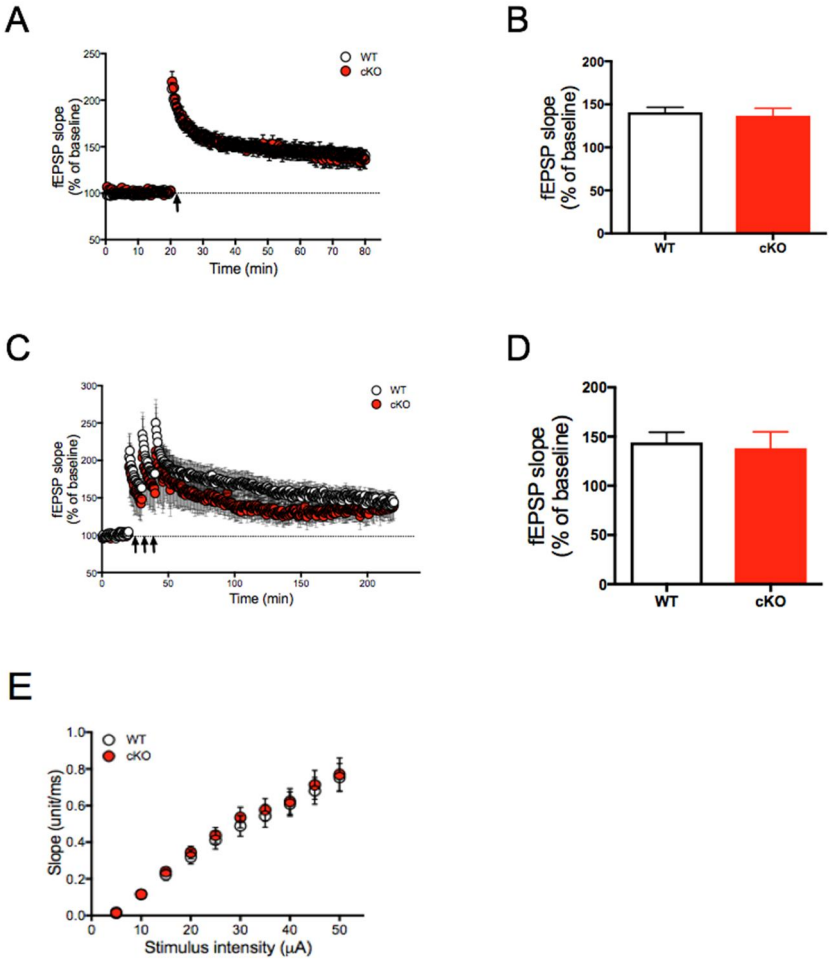
(C, D) In the recent memory probe test of the MWM test, CTCF cKO mice performed comparably to WT (Figure 5C; two-way ANOVA, effect of interaction, $p = 0.2976$; Figure 5D; WT, 3.071 ± 0.5593; cKO, 1.818 ± 0.5191; unpaired *t*-test; $p = 0.1225$; TQ: target quadrant, OQ: opposite quadrant, AQ: adjacent quadrant). (E, F) When the same mice were tested 3 weeks after training, CTCF cKO mice displayed loss of spatial memory with less number of platform crossing and higher mean distance to the platform (Figure 5E; two-way ANOVA, effect of interaction, $p = 0.175$; Figure 5F; WT, 5.429 ± 0.7317; cKO, 2.364 ± 0.4724; unpaired *t*-test; $p = 0.0031$).

(G) Swimming speed during recent probe test (WT, n=14; 20.89 ± 0.7795 cm/s; cKO, n=11; 18.04 ± 1.063 cm/s; unpaired *t*-test; $*p = 0.0370$).

(H) Swimming speed during remote probe test (WT, n=14; 21.17 ± 0.6098 cm/s; cKO, n=11; 17.16 ± 0.6548 cm/s; unpaired *t*-test; $***p = 0.0002$).

Impaired cortical synaptic plasticity in CTCF cKO mice

Next, I performed electrophysiological experiments to verify the underlying mechanisms of the remote memory deficit at the synaptic level. I chose hippocampal and ACC field recordings to compare the contribution of hippocampal and cortical LTP in the remote memory process. In the hippocampal slice recording, SC-CA1 E-LTP and L-LTP were induced normally and the potential levels stably lasted for 1 h and 3 h, respectively (Figures 9A–D). I performed input-output curve (IO curve) and confirmed that hippocampal basal transmission is normal in CTCF cKO mice (Figure 9E). Together, these results suggested that the hippocampal deletion of CTCF does not affect the electrophysiological properties. Since these results are in line with the normal recent memory shown in CTCF cKO mice, I tested the cortical plasticity, which is a physiological trace of remote memory (Frankland and Bontempi, 2005). In the CTCF cKO ACC slices, E-LTP was normal, while L-LTP appeared significantly impaired with a considerably lower potentiation level (Figures 10A–D). Moreover, the basal transmission was also downregulated in CTCF cKO mice (Figure 10E), indicating that CTCF has a region-specific role in regulating the cortical basal transmission level. The slice recording results indicated that CTCF deletion specifically disrupts the cortical synaptic plasticity, which leads to impaired performance in remote memory behaviors. These results provided physiological evidence of the shift from the hippocampus to the cortex as a core region in processing the remote memory and showed that the regulation of the cortical chromatin structure by CTCF is necessary for memory consolidation.


A
WT
cKO
fEPSP slope
(% of baseline)
Time (min)
B
fEPSP slope
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WT
cKO
C
WT
cKO
fEPSP slope
(% of baseline)
Time (min)
D
fEPSP slope
(% of baseline)
WT
cKO
E
WT
cKO
Slope (unit/ms)
Stimulus intensity (μA)

Figure 9. CTCF cKO mice have intact hippocampal synaptic plasticity

(A, B) Hippocampal E-LTP was normal in CTCF cKO mice (WT, n = 13; cKO, n = 8; average of fEPSP slopes for the last 5 min; WT, 140.8 ± 5.8%; cKO, 136.8 ± 8.6%; unpaired *t*-test; $p = 0.6947$).

(C, D) CTCF cKO mice did not exhibit any impairment in the TBS-induced hippocampal L-LTP and the potentiation level was maintained for 3 h at a comparable level to WT (WT, n = 5; cKO, n = 4; average fEPSP slopes for the last 10 min; WT, 144.0 ± 10.4%; cKO, 138.1 ± 16.7%; unpaired *t*-test; $p = 0.7635$).

(E) IO curve was normal in CTCF cKO mice (WT, n = 11; cKO, n = 8; repeated measure two-way ANOVA, effect of genotype, $F_{1,17} = 0.0929$; $p = 0.7641$).

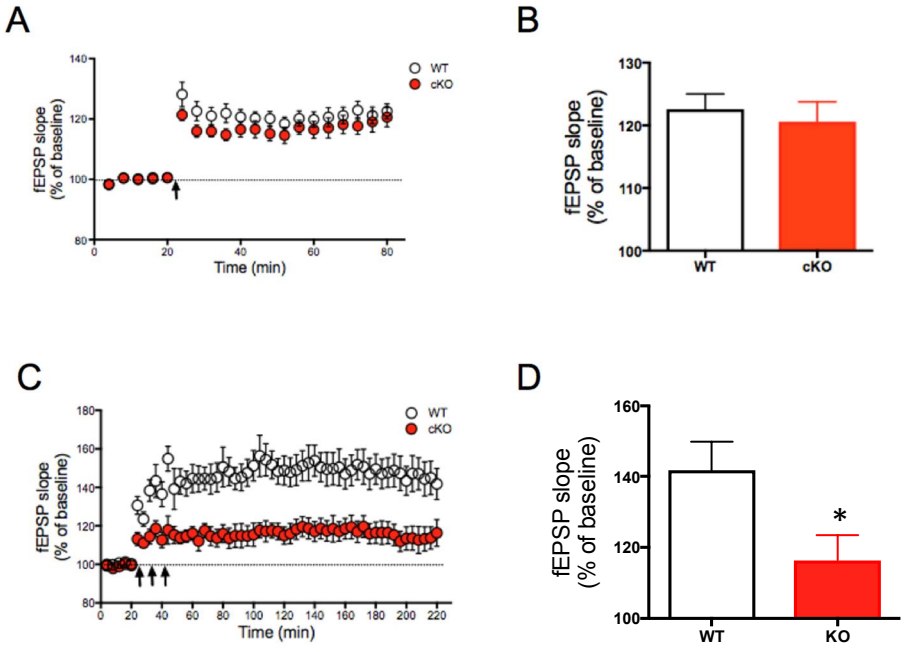
A
fEPSP slope (% of baseline)
Time (min)
WT
cKO
B
fEPSP slope (% of baseline)
WT
cKO
C
fEPSP slope (% of baseline)
Time (min)
WT
cKO
D
fEPSP slope (% of baseline)
WT
KO
*
E
Slope (unit/ms)
Stimulus intensity (μA)
WT
cKO
*

Figure 10. CTCF cKO mice display impaired protein synthesis-dependent form of cortical synaptic plasticity.

(A, B) CTCF cKO mice displayed normal cortical E-LTP (WT, n = 16; cKO, n = 12; average of fEPSP slopes for the last 4 min; WT, 122.6 ± 2.5%; cKO, 120.6 ± 3.2%; unpaired *t*-test; $p = 0.6190$).

(C, D) CTCF cKO mice showed a significant deficit in cortical L-LTP with a substantially decreased potentiation level after induction (WT, n = 10; cKO, n = 6; average fEPSP slopes for the last 8 minutes; WT, 141.7 ± 8.1%; cKO, 116.4 ± 6.9%; unpaired *t*-test; $p = 0.0498$).

(E) Cortical basal transmission was attenuated in CTCF cKO mice (WT, n = 8; cKO, n = 8; repeated measure two-way ANOVA, effect of genotype, $F_{1,14} = 7.364$; $p = 0.0168$).

Gene expression profile changes upon CTCF deletion in cortical neuron culture

To investigate the molecular changes underlying the phenotypes of the CTCF cKO and HT mice, I explored the variations in the gene expression profile. It is a well-established thought that appropriate gene expression is necessary for memory (Igaz et al., 2004; Peixoto and Abel, 2013). Since CTCF regulates transcription through chromatin remodeling, I expected that CTCF deletion would lead to drastic changes in the gene expression. I induced CTCF knockdown (KD) in primary mouse cortical cultures using adeno-associated virus (~67% mRNA reduction) and performed RNA sequencing (RNA-seq). I found a total of 394 differentially expressed genes (DEGs) in CTCF KD neurons with the fold change cut off set to >1.5. Of the 394 DEGs, 146 (37%) genes were upregulated, while 248 (63%) genes were downregulated. This indicated a shift in the overall gene expression to a downregulating direction (Figure 11), which can be explained by the reduced CTCF transcriptional activity. These results were also in line with previous results of CTCF deletion studies (Hirayama et al., 2012; Wan et al., 2008). Before further analysis, I performed quantitative reverse-transcription PCR (qRT-PCR) experiments using the ACC tissues from CTCF cKO mice and ratified that the effects of CTCF deletion in the cortical culture are similar to those of CTCF deletion *in vivo* (Figure 13A–E). To analyze the DEGs from RNA-seq with unifying terminologies, I performed GO analysis, which is a major bioinformatics technique that uses gene annotations to hierarchically classify the genes and their functions (Ashburner et al., 2000). I found that

many of the DEGs are involved in cell adhesion, neuroactive ligand-receptor interaction, and calcium binding. More importantly, I found that genes that are involved in learning and memory, synapse assembly, and cognition were enriched in the DEG list (Table 1), suggesting that they were inappropriately expressed in the CTCF deficient cells. For more detailed investigation, I performed IPA to connect the DEGs in pathways under certain terminologies, and I found that the IPA results exactly reflected the behavioral and electrophysiological phenotypes of the CTCF cKO and HT mice. Memory and learning were predicted to be functionally inhibited, and, specifically, long-term memory and memory consolidation appeared to be inhibited (Figures 12B, C). The expression of most of the DEGs functionally involved in LTP and synaptic transmission were decreased, leading to the inhibition of the two functions (Figures 12D, E). Furthermore, in the DEG list, I found a highly frequent appearance of the clustered *Pcdh* family isoforms. There was a total of 27 *Pcdh* genes, which made up 10% of the DEG list (Table 2). This pattern was similar to the previous microarray analysis data from Hirayama et al. (Hirayama et al., 2012), indicating that CTCF critically regulates the neuronal *Pcdh* expression in adult as well as developmental brain. Taken together, the RNA-seq data and bioinformatics analysis suggested that CTCF deletion impairs chromatin remodeling and alters the expression of target genes, which leads to defects in the cortical plasticity and remote memory in CTCF-deficient mice.

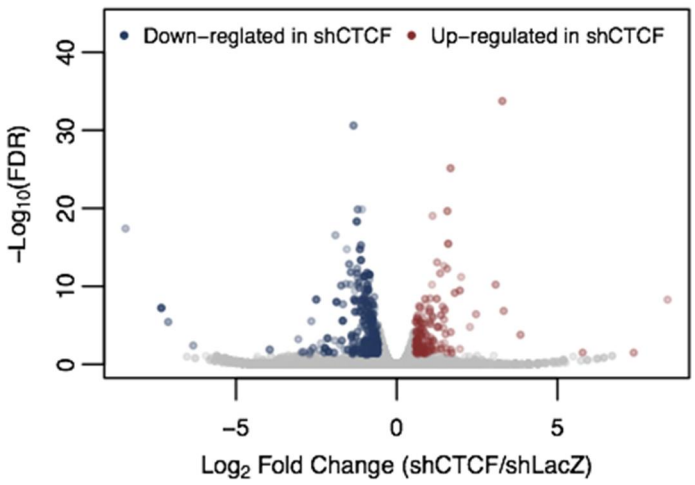

Figure 11. Volcano plot of DEGs

The volcano plot shows that more number of genes were downregulated than upregulated in the DEG list from the RNA-seq data of CTCF KD cortical culture.

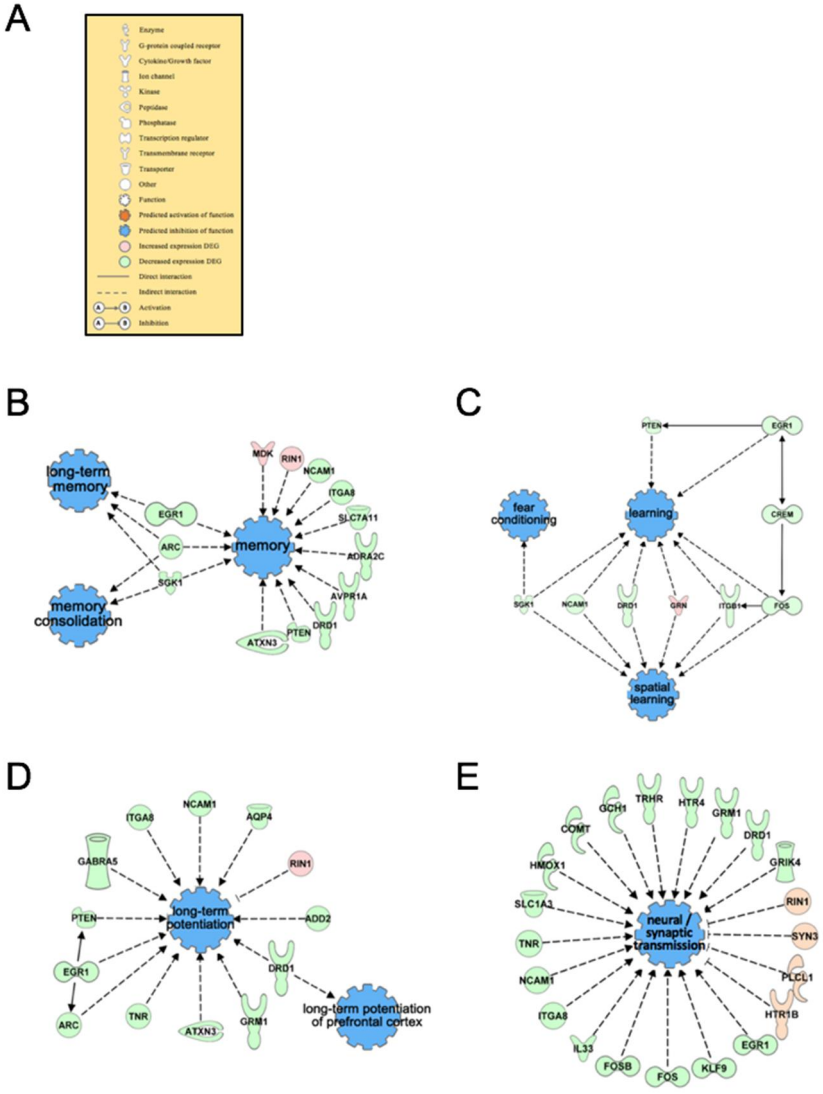
A
Enzyme
G-protein coupled receptor
Cytokine/Growth factor
Ion channel
Kinase
Peptidase
Phosphatase
Transcription regulator
Transmembrane receptor
Transporter
Other
Function
Predicted activation of function
Predicted inhibition of function
Increased expression DEG
Decreased expression DEG
Direct interaction
Indirect interaction
Activation
Inhibition
B
long-term memory
memory consolidation
memory
EGR1
ARC
SGK1
MDK
RIN1
NCAM1
ITGA8
SLC7A11
ADRA2C
AVPR1A
DRD1
PTEN
ATXN3
C
fear conditioning
learning
spatial learning
PTEN
EGR1
CREM
SGK1
NCAM1
DRD1
GRN
ITGB1
FOS
D
long-term potentiation
long-term potentiation of prefrontal cortex
ITGA8
NCAM1
AQP4
GABRA5
RIN1
PTEN
ADD2
EGR1
ARC
TNR
ATXN3
GRM1
DRD1
E
neural / synaptic transmission
GCH1
TRHR
HTR4
GRM1
DRD1
COMT
HMOX1
GRIK4
SLC1A3
RIN1
TNR
SYN3
NCAM1
PLCL1
ITGA8
HTR1B
IL33
EGR1
FOSB
FOS
KLF9

Figure 12. IPA analysis of DEGs from the RNA-seq data

(A) Legend for IPA analysis.

(B–E) IPA showed connections of DEGs for four functions: memory, learning, long-term potentiation, and synaptic transmission. All four functions were all predicted to be inhibited (blue).

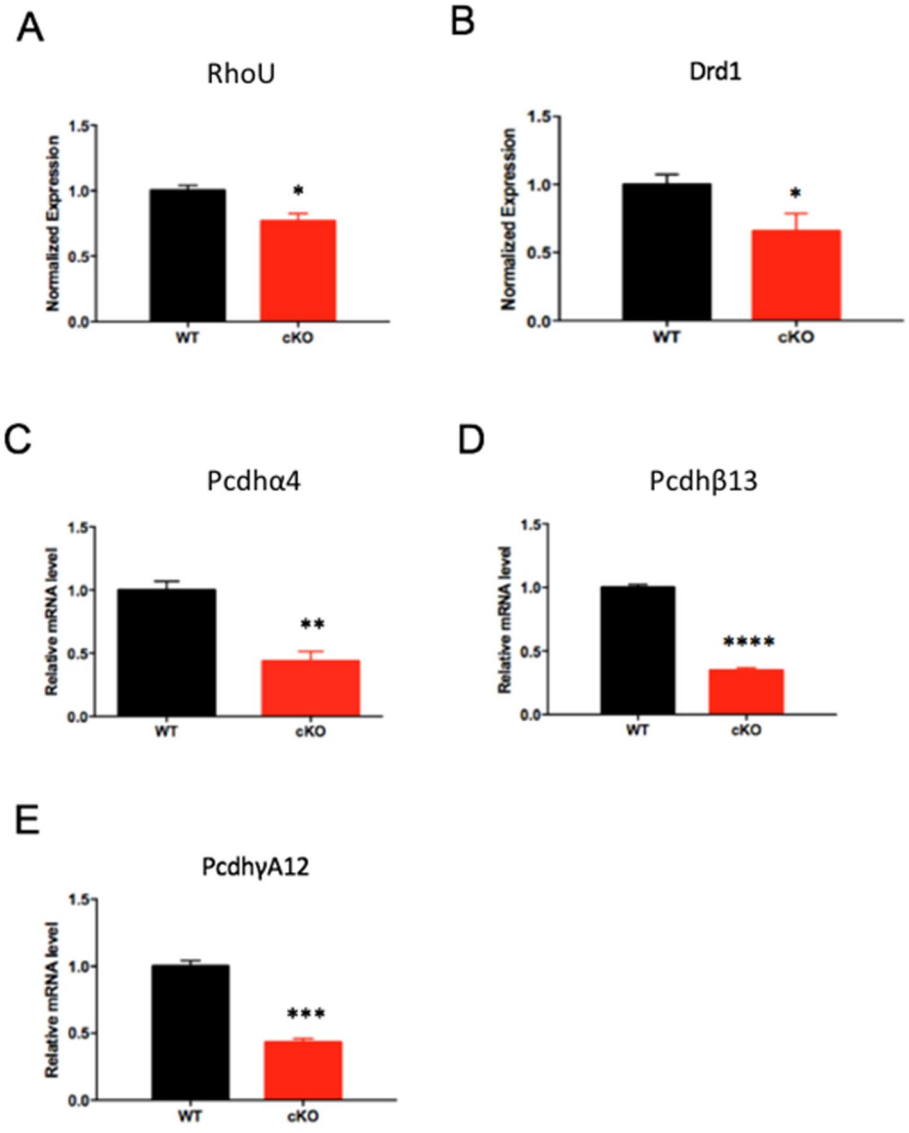
A
RhoU
Normalized Expression
1.5
1.0
0.5
0.0
*
WT
cKO
B
Drd1
Normalized Expression
1.5
1.0
0.5
0.0
*
WT
cKO
C
Pcdhα4
Relative mRNA level
1.5
1.0
0.5
0.0
**
WT
cKO
D
Pcdhβ13
Relative mRNA level
1.5
1.0
0.5
0.0
****
WT
cKO
E
PcdhγA12
Relative mRNA level
1.5
1.0
0.5
0.0
***
WT
cKO

Figure 13. qRT-PCR of ACC tissues from CTCF cKO mice confirm the RNA-seq data of gene downregulation

(A) Normalized expression of RhoU mRNA (WT, n = 4; cKO, n = 3; WT, 1 ± 0.04013%; cKO, 0.7669 ± 0.05816%; unpaired *t*-test; $p = 0.0187$).

(B) Normalized expression of Drd1 mRNA (WT, n = 4; cKO, n = 3; WT, 1 ± 0.07316%; cKO, 0.6552 ± 0.1332%; unpaired *t*-test; $p = 0.0584$).

(C) Normalized expression of Pcdhα4 mRNA (WT, n = 4; cKO, n = 3; WT, 1 ± 0.06878%; cKO, 0.4386 ± 0.07655%; unpaired *t*-test; $p = 0.0029$).

(D) Normalized expression of Pcdhβ13 mRNA (WT, n = 4; cKO, n = 3; WT, 1 ± 0.02351%; cKO, 0.3466 ± 0.02203%; unpaired *t*-test; $p < 0.0001$).

(E) Normalized expression of PcdhγA12 mRNA (WT, n = 4; cKO, n = 3; WT, 1 ± 0.04239%; cKO, 0.4336 ± 0.02648%; unpaired *t*-test; $p = 0.0001$).

Category	ID	Term	q-value	# of genes
GO: Biological Process	GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	2.84E-17	29
GO: Biological Process	GO:0098742	cell-cell adhesion via plasma-membrane adhesion molecules	6.90E-17	33
GO: Molecular Function	GO:0005509	calcium ion binding	3.36E-12	48
GO: Biological Process	GO:0016339	calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules	8.26E-10	12
GO: Biological Process	GO:0098609	cell-cell adhesion	3.96E-07	48
GO: Biological Process	GO:0007155	cell adhesion	4.83E-07	63
GO: Biological Process	GO:0022610	biological adhesion	5.50E-07	63
GO: Biological Process	GO:0007416	synapse assembly	5.33E-05	15
GO: Cellular Component	GO:0005887	integral component of plasma membrane	8.72E-04	55
GO: Cellular Component	GO:0031226	intrinsic component of plasma membrane	8.72E-04	56
GO: Cellular Component	GO:0034678	integrin alpha8-beta1 complex	8.72E-04	3
GO: Biological Process	GO:0051482	positive regulation of cytosolic calcium ion concentration involved in phospholipase C-activating G-protein coupled signaling pathway	3.70E-03	5
GO: Biological Process	GO:0007611	learning or memory	4.81E-03	17
GO: Biological Process	GO:0050808	synapse organization	9.99E-03	16
GO: Biological Process	GO:0060322	head development	1.11E-02	32
GO: Biological Process	GO:0007610	behavior	1.38E-02	28
GO: Biological Process	GO:0050890	cognition	1.38E-02	17
GO: Biological Process	GO:0044708	single-organism behavior	1.43E-02	23
GO: Cellular Component	GO:0098636	protein complex involved in cell adhesion	1.56E-02	5
GO: Cellular Component	GO:0008305	integrin complex	1.56E-02	5
GO: Biological Process	GO:0050905	neuromuscular process	1.86E-02	10
GO: Biological Process	GO:0007268	chemical synaptic transmission	2.81E-02	27
GO: Biological Process	GO:0099537	trans-synaptic signaling	2.81E-02	27
GO: Biological Process	GO:0098916	anterograde trans-synaptic signaling	2.81E-02	27
GO: Biological Process	GO:0007267	cell-cell signaling	2.81E-02	41
GO: Biological Process	GO:0007420	brain development	2.85E-02	29
GO: Biological Process	GO:0099536	synaptic signaling	3.02E-02	27
GO: Biological Process	GO:0031644	regulation of neurological system process	3.45E-02	8
GO: Biological Process	GO:0006575	cellular modified amino acid metabolic process	3.62E-02	14
GO: Cellular Component	GO:0098589	membrane region	4.37E-02	39
GO: Biological Process	GO:1902475	L-alpha-amino acid transmembrane transport	4.49E-02	5
GO: Biological Process	GO:0033555	multicellular organismal response to stress	4.81E-02	8
GO: Biological Process	GO:0031589	cell-substrate adhesion	4.81E-02	16
GO: Molecular Function	GO:1901681	sulfur compound binding	4.82E-02	15

Table 1. GO term list

List of the GO terms and categories in order of the smallest to the largest q-value. The far-left column shows the number of DEGs that fall into each GO term.

ENSEMBL_ID	Gene_symbol	Foldchange	FDR
ENSMUSG00000104252	Pcdha4	2.79603809	8.1255E-06
ENSMUSG00000104318	Pcdha7	1.75131089	3.3856E-07
ENSMUSG00000103770	Pcdha9	1.50533694	0.0170364
ENSMUSG00000051599	Pcdhb2	1.57070315	0.03101376
ENSMUSG00000045498	Pcdhb3	1.55631839	0.02999433
ENSMUSG00000045689	Pcdhb4	1.625148	0.00566807
ENSMUSG00000063687	Pcdhb5	2.09150871	4.5222E-09
ENSMUSG00000045062	Pcdhb7	1.5490714	0.00072286
ENSMUSG00000051242	Pcdhb9	2.21286193	1.8998E-15
ENSMUSG00000045657	Pcdhb10	1.65599862	0.00078325
ENSMUSG00000051486	Pcdhb11	1.88906083	6.1688E-09
ENSMUSG00000043458	Pcdhb12	2.19555803	2.6991E-07
ENSMUSG00000047307	Pcdhb13	2.44499604	9.4975E-09
ENSMUSG00000047033	Pcdhb15	1.53833238	0.04003896
ENSMUSG00000047910	Pcdhb16	1.68348686	2.4564E-06
ENSMUSG00000046387	Pcdhb17	1.5461865	0.00016953
ENSMUSG00000048347	Pcdhb18	1.65724535	3.5439E-06
ENSMUSG00000043313	Pcdhb19	1.90074633	1.314E-11
ENSMUSG00000046191	Pcdhb20	1.62866281	8.2162E-05
ENSMUSG00000044022	Pcdhb21	2.14201216	2.8263E-08
ENSMUSG00000073591	Pcdhb22	1.8448699	3.0912E-10
ENSMUSG00000103144	Pcdhga1	1.8666182	3.0912E-10
ENSMUSG00000103332	Pcdhga2	1.65288697	5.3454E-07
ENSMUSG00000102440	Pcdhga9	1.50955405	0.00326747
ENSMUSG00000102428	Pcdhga12	2.02084532	6.2239E-12
ENSMUSG00000103037	Pcdhgb1	1.75647878	4.0811E-06
ENSMUSG00000050505	Pcdh20	1.57880867	0.00362815

Table 2. List of the downregulated *Pcdh* isoforms

In the gene expression analysis, CTCF KD neurons exhibited a total number of 27 *Pcdh* isoforms with decreased expression. Fold changes indicate the degree of the expressional downregulation. Of the clustered *Pcdh* isoforms, 18 *Pcdhβ* (66%), 5 *Pcdhγ* (19%), and 3 *Pcdhα* (11%) were found. There was one non-clustered *Pcdh* isoform.

DISCUSSION

In this chapter, I showed that CTCF deletion in excitatory neuron causes remote memory-specific deficits. These results suggest that CTCF-mediated chromatin remodeling and transcription are important for memory consolidation, through which memory traces are stabilized for long-term storage. Since remote memory is believed to be stored in cortex, I measured the level of long-term potentiation in the CTCF cKO mice. As a result, I found that CTCF cKO mice exhibit impaired cortical synaptic plasticity, which is in line with the behavioral phenotypes shown earlier. I also showed that CTCF is necessary for appropriate gene expression, as a number of genes involved in learning and memory-related process were differentially expressed in the CTCF KD group. This chapter provides behavioral, physiological, molecular evidence for the involvement of CTCF in the remote memory and elucidates our understanding of systems consolidation mechanisms.

In this study, I found that cortical synaptic transmission is decreased in the CTCF cKO mice. Basal synaptic transmission in slice field recording can be measured by several methods, including input-output curve (IO curve) and paired-pulse ratio (PPR) (Madronal, 2009). IO curve experiment is performed by giving stimuli at different intensities (for example, 5-50uA), which account for the “input”. Then, the changes in the response size are measured, which account for the “output”. Often, changes in the synaptic transmission are discussed in relation to altered basal neural excitability and behavioral sensitization of the animal. For example, Beurrier et al. (Beurrier

and Malenka, 2002) have shown that inhibitory effects of dopamine (DA) on nucleus accumbens (NAc) synaptic transmission are increased during behavioral sensitization to cocaine. In other words, after chronic exposure to cocaine, DA-induced inhibition of excitatory synaptic transmission is increased, contributing to molecular changes after drug abuse. Also, Cuevas-Olguin et al. (Cuevas-Olguin et al., 2017) recently reported that pro-inflammatory cytokine interleukin 6 (IL-6) is involved in the regulation of synaptic transmission. The absence of IL-6 caused an increase of basal excitability in the prefrontal cortex and sensitivity to pentylenetetrazole (PTZ)-induced seizures. As synaptic transmission depends on several molecular components, it is known to be related to excitatory AMPA receptor trafficking (Freche et al., 2012). Many neurological disorders such as autism, mental retardation, Alzheimer's disease, and addiction are known to be related to altered synaptic transmission (van Spronsen and Hoogenraad, 2010). Since the CTCF cKO mice showed cortex-specific downregulation of basal synaptic transmission, it would be meaningful to further examine CTCF's role in areas that are connected to ACC and assess CTCF's participation in the pathology of neurological disorders mentioned above.

CHAPTER III

Function of CTCF in forebrain inhibitory neurons and CTCF deletion-induced neurodegeneration in aged mice

INTRODUCTION

After assessing CTCF's role in excitatory neurons, I next turned to exploring CTCF's role in inhibitory neurons. Although cortical inhibitory neurons comprise only 10-15% of the entire neuron population, they play crucial roles in regulating neuronal excitability (Gaykema et al., 2014). Inhibitory neurons are also called interneurons, and they tend to have high firing rates and abilities to produce the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Gaykema et al., 2014). Interneuron populations are heterogeneous and include parvalbumin (PV)- and somatostatin (SOM)-expressing neurons. GABAergic neurons are involved in shaping overall rhythmic activity and the output of the excitatory cells. Therefore, inhibitory neurons are important for keeping the excitation-inhibition balance in the brain. The imbalance in the excitation-inhibition is implicated in various cognitive disorders, such as Autism Spectrum Disorders (ASD) and Schizophrenia (SCZ) (Gao and Penzes, 2015; Nelson and Valakh, 2015). While glutamatergic neurons mostly form synapses onto dendritic spines, GABAergic neurons form synapses onto dendritic shaft, somata, and axon initial segments (Gao and Penzes, 2015).

In this chapter, I also examined CTCF's function in relation to neurodegeneration and gliosis. Gliosis refers to responsive changes by glial cells upon tissue or cell damage (Burda and Sofroniew, 2014). Often, gliosis is accompanied by proliferation of different type of glial cells, such as astrocytes, microglia, and oligodendrocytes (Pekny and Nilsson, 2005).

Gliosis is composed of cascades of molecular processes with various gene induction. Increased expression of transforming growth factor β (TGF-β), interleukins, inflammatory cytokines interferon- γ (IFN- γ), and fibroblast growth factor (FGF2) are known to be important for trigger of gliosis. (Pekny and Pekna, 2016) Among the interleukins, interleukin-1 (IL-1) is especially known to initiate inflammatory response of astrocytes. Since gliosis is a nonspecific responsive reaction, it is implicated in many different types of injuries and diseases. Gliosis is observed in acute conditions, like ischemia and stroke (Pekny and Pekna, 2016). Moreover, gliosis is also observed in pathogenesis of central nervous system (CNS) diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease. Gliosis can potentially alter cellular activities and cause functional changes in neuronal and non-neuronal cells. In healthy CNS, glial cells are highly active and dynamic. Astrocytes help regulate homeostasis of CNS by recycling neurotransmitters and providing nutrients to neurons (Ricci et al., 2009). Astrocytes are also known to be important for synaptic plasticity and adult neurogenesis. Microglia critically regulate synapse development and turnover (Eyo et al., 2016). Reactive gliosis refers to multicellular response to CNS insults, accompanied by functional and morphological changes of glial cells. While reactive gliosis is a highly context-dependent process, it aims to restore homeostasis and minimize the progression of tissue damage (Pekny and Nilsson, 2005). Dysfunction of gliosis can have detrimental effect on neural synaptic plasticity. Neurodegeneration is a progressive loss of nerve structure and function (Przedborski et al., 2003). Neurodegenerative disorders are

characterized by gradual cell death, which can eventually cause physical and mental dysfunction in patients. Neurodegeneration can arise from many factors, including genetic mutations and environmental influences like oxidative stress and toxicant exposure (Amor et al., 2010).

EXPERIMENTAL PROCEDURES

Animals

CTCF HT mice were generated by crossing CTCFfl/+ with VgatCre/+. Littermates that did not carry the Cre transgene or the floxed CTCF were used as controls. 12- to 15-week old adult male and female mice were used for the molecular, behavioral, and electrophysiological experiments. All animals were housed under a 12-h light/dark cycle with food and water provided *ad libitum*. The Animal Care and Use Committee of Seoul National University approved the animal protocols.

Behavioral Tests

Male mice at 12–15 weeks of age were used for the behavioral analyses in this study. The behavioral experiments were performed essentially following our previous study (Kim et al., 2016). For the Morris water maze, mice were handled daily for 3 min over a week before training. The water maze was a gray cylinder-shaped tank (140 cm diameter, 100 cm height) placed in a room with multiple spatial cues and dim light. Water mixed with white paint (19–21 °C) was filled up to 1 cm above the escape platform (10 cm diameter). Mice were trained for four trials per day with 1 min intertrial intervals. Mice were placed into the different edge points of the maze in each four trials, facing the inner wall of the tank, and tracked using the Ethovision software (Noldus). The order of releasing point was changed daily. When mice reached

the platform within 60 s, they were removed from the maze and returned to the transport cage. When they failed, they were guided to or placed on the platform and were subsequently removed from the maze. After 5 days of training, a probe test was performed after removing the platform. Mice were placed at the center of the maze and tracked for 1 min. One more session of training was performed after the probe test. To check the remote memory, the probe test was performed 4 weeks after the last training. For the contextual fear conditioning, mice were placed into the Coulbourne fear conditioning chamber. After 148 s, they received foot shocks (2 s, 0.75 mA) twice with 30 s interval. After 30 s, they were returned to the home cage. Contextual fear memory was tested by placing the mice again in the conditioning chamber and measuring the freezing levels (immobility) for 4 min using Freeze Frame software (Coulbourne).

Electrophysiology

Hippocampal field excitatory postsynaptic potential (fEPSP) recording

Field excitatory postsynaptic potential (fEPSP) recordings were performed as described previously (Park et al., 2014). After anesthetization with isoflurane, mice were decapitated and their brains were removed. Transverse hippocampal slices were sectioned 400 μm thick using a vibratome (Leica, Hesse, Germany). The slices were retained at 32 °C for 30 min during the recovery period and then incubated at 28 °C until the experiment. All incubation chambers were submerge-fashioned and the artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 2.5 mM KCl, 1 mM

NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 2.6 mM $CaCl_2$, 1.3 mM $MgSO_4$) was oxygenated with 95% O_2 and 5% CO_2 and perfused at 1 ml/min throughout the experiment. fEPSPs were recorded from the Schaffer collaterals (SC) of CA1. Stimuli were given every 30 sec using concentric bipolar electrodes (MCE-100; Kopf Instruments) and the responses were recorded using a glass pipette electrode filled with ACSF (1 MΩ). Field potentials were amplified, low-pass filtered (GeneClamp 500; Axon Instruments), and then digitized (NI PCI-6221; National Instruments) for measurement. Data were monitored, analyzed online, and reanalyzed offline using the WinLTP program (WinLTP Ltd., winltp.com, The University of Bristol, UK). For the LTP and LTD experiments, stimulation was provided at the intensity that produces roughly 40% of the slice's maximum slope. Two responses elicited per minute were averaged and expressed relative to an average of the 20-min baseline responses. Theta burst stimulation (TBS) protocols were used to induce E-LTP and L-LTP (five pulses of 100 Hz repeated five times at 5 Hz; 10 s inter-train interval used for E-LTP; 10 min inter-train interval for L-LTP). The fEPSP response average of the last 5 and 10 min of the E-LTP and L-LTP experiments were used to compare the level of synaptic plasticity between the groups.

ACC field potential recording using multi-electrode array

For ACC multi-electrode array experiments, three to four 300-μm thick coronal brain slices after the corpus callosum connection were sectioned using a vibratome. The slices were incubated in a submerged chamber at room

temperature until the experiment. ACSF (124 mM NaCl, 2.5 mM KCl, 1 mM NaH2PO4, 25 mM NaHCO3, 10 mM glucose, 2.5 mM CaCl2, 1 mM MgSO4) was oxygenated with 95% O_2 and 5% CO_2 and perfused at 2–3 ml/min throughout the experiment. The MED64 system (Parasonic, Osaka, Japan) was used as previously described (Kang et al., 2012). A slice was placed on the MED64 probe (MED-P515A, 8 × 8 array, interpolar distance 150 μm, Parasonic) and perfused with ACSF at 28–30 °C. The electrical stimulation (1–20 μm, 0.2 ms) was given to a channel in the deep layer region. MED64 Mobius was used for data acquisition and analysis. One pulse was given per minute and the data were averaged every 4 min. The percentages of the last 4 min (E-LTP) and 8 min (L-LTP) fEPSP slopes were normalized to the averaged value of the 20-min baseline.

Histology, immunohistochemistry, and imaging

Mice were anesthetized by isoflurane and transcardially perfused with 4% paraformaldehyde (PFA) in PBS for tissue fixation. Brains were kept in the PFA solution at 4°C overnight for further fixation. Then, brains were moved to 30% PBS-based sucrose solution for 2 days for dehydration. After the dehydration is completed, brains were frozen and cut into coronal slices using cryostat (Leica Ltd., Germany). Hippocampus was cut into 40μm thick slices, while ACC was cut into 30μm thick slices. Brain slices were then washed with PBS, blocked with blocking solution, and incubated with primary and secondary antibodies consecutively. After the antibody

application, the slices were mounted on slide glasses with 50% PBS-based glycerol solution for imaging.

Fluorescence images were acquired using a confocal microscope and analyzed with ImageJ program.

Experimental Design and Statistical Analysis

All experiments were performed in a blind fashion. I used the minimum number of mice that can produce statistical validity. I used 8-16 mice for behavioral tests, 6-10 for electrophysiology, and 2-4 for IHC experiment. There were no sex-related differences observed in the CTCF HT and CTCF cKO mice, so both male and female mice were used for the experiments.

Data were represented by the mean ± standard error of the mean (SEM). For each variable, comparison of two groups were made using student's t-test. Two-way ANOVA and *post-hoc* Bonferroni test were used for further comparisons. The statistical significance level was set at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

CTCF in forebrain inhibitory neurons also participate in remote memory maintenance

Having found that the CTCF deletion in excitatory neurons induces remote memory deficits, I further examined the effect of CTCF deletion in the inhibitory neurons. I crossed the floxed CTCF line with the *Vgat-Cre* line to induce a forebrain inhibitory neuron-specific deletion of CTCF during development. However, I found that homozygous CTCF KO mice were embryonic lethal and only heterozygous mice survived. I concluded that as in the previous report (Hirayama et al., 2012), the homozygous deletion of CTCF in inhibitory neurons during development has a lethal effect on the animals and confirmed that CTCF is crucial for development. I used the heterozygous CTCF mice (CTCF HT) for the experiments. As with the CTCF cKO mice, I trained the CTCF HT mice in the MWM and CFC and examined recent and remote memory. In the CFC, the CTCF HT mice exhibited normal freezing level in both recent and remote memory tests (Figure 14A). However, in the MWM, the CTCF HT mice displayed a significant impairment in finding the platform with a higher number of platform crossings in the probe test (Figures 14E–G). The recent spatial memory was completely intact in the CTCF HT mice (Figures 14B–D). These results indicated that the CTCF HT mice have a deficit in spatial remote memory and that CTCF in the forebrain inhibitory neurons is also involved in systems consolidation. The partial

impairment may be due to an assumedly mild effect of the heterozygous CTCF deletion compared to the homozygous deletion. In the extracellular field recording, the basal transmission and LTP appeared normal in both hippocampus (Figures 15A-C) and ACC (Figures 16A–E) of the CTCF HT mice, which was predictable because there exist far more excitatory neurons than inhibitory neurons in the ACC and the field recording measures the field excitatory postsynaptic potential (fEPSP).

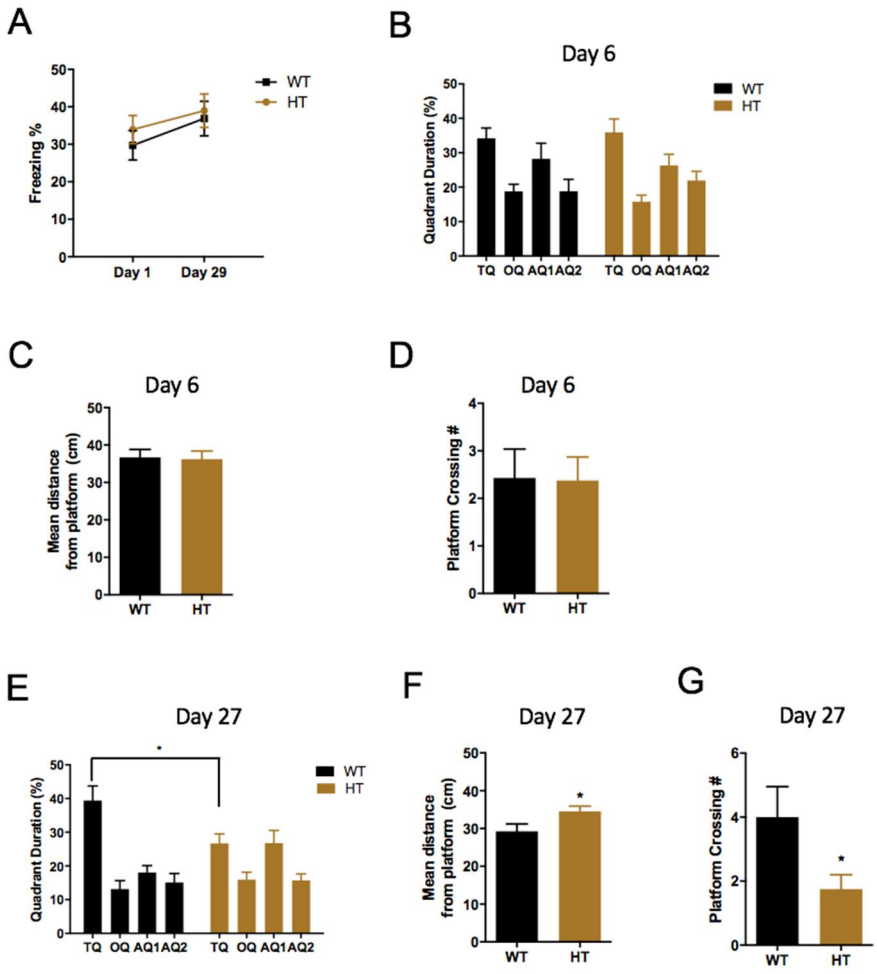
A
Freezing %
WT
HT
Day 1
Day 29
B
Day 6
Quadrant Duration (%)
TQ OQ AQ1 AQ2 TQ OQ AQ1 AQ2
C
Day 6
Mean distance from platform (cm)
D
Day 6
Platform Crossing #
E
Day 27
Quadrant Duration (%)
F
Day 27
Mean distance from platform (cm)
G
Day 27
Platform Crossing #

Figure 14. CTCF HT mice show a partial behavioral deficit in remote memory

(A) CFC recent and remote memory were normal in CTCF HT mice (WT, n = 15; HT, n = 16; two-way ANOVA; effect of interaction, $p = 0.8026$).

(B–D) CTCF HT mice performed comparably to WT in the recent memory test of the MWM (WT, n = 7; HT, n = 8; Figure 10B; two-way ANOVA; effect of interaction, $p = 0.7411$; Figure 10C; WT, 36.7 ± 2.126; cKO, 36.24 ± 2.155; unpaired *t*-test; $p = 0.8835$; Figure 10D; WT, 2.429 ± 0.6117; HT, 2.375 ± 0.4978; unpaired *t*-test; $p = 0.9463$).

(E–G) 3 weeks later, the CTCF HT mice showed a significant memory deficit in the MWM probe test. Quadrant duration, distance from platform, and number of platform crossings were all impaired (WT, n = 7; HT, n = 8; Figure 10E; two-way ANOVA; effect of interaction, $p = 0.046$; Bonferroni *post-hoc* test for TQ; $p = 0.0124$; Figure 10F; WT, 29.23 ± 1.985; HT, 34.55 ± 1.372; unpaired *t*-test; $p = 0.0423$; Figure 10G; WT, 4 ± 1.750; HT, 1.750 ± 0.4532; unpaired *t*-test; $p = 0.0442$).

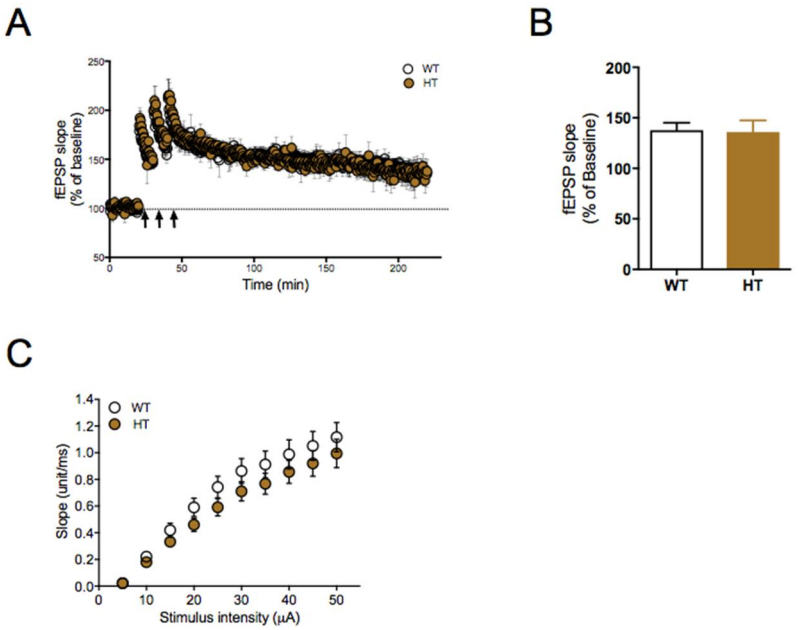

Figure 15. CTCF HT mice have normal hippocampal synaptic plasticity

(A, B) TBS-induced L-LTP in CTCF HT mice was maintained at a comparable level to that of WT for three hours mice (WT, n = 7; HT, n = 7; average of fEPSP slopes for the last 10 min; WT, 137.8 ± 7.2%; HT, 135.8 ± 11.6%; unpaired *t*-test; $p = 0.8874$).

(C) Hippocampal basal transmission measured through IO curve appeared normal in CTCF HT mice (WT, n = 13; cKO, n = 14; repeated measure two-way ANOVA, effect of genotype, $F_{1,25} = 1.312$; $p = 0.2629$).

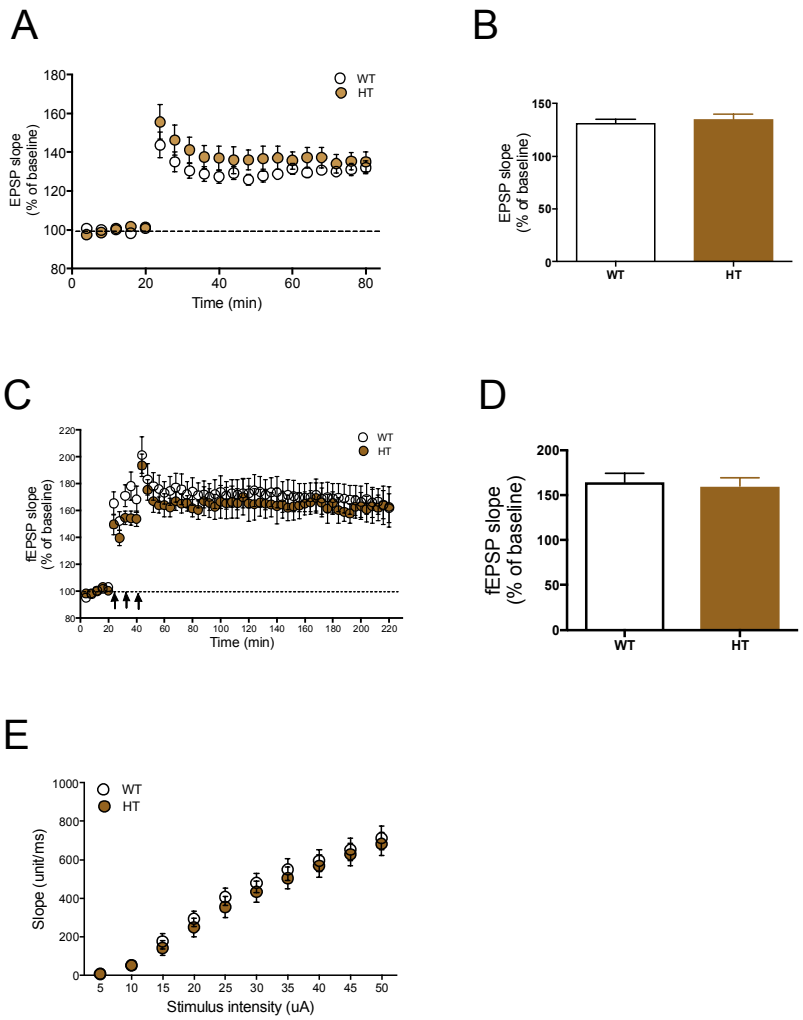
A
B
C
D
E
WT
HT
EPSP slope
(% of baseline)
Time (min)
fEPSP slope
(% of baseline)
Slope (unit/ms)
Stimulus intensity (uA)

Figure 16. CTCF HT mice show normal level of synaptic plasticity in ACC

(A, B) E-LTP was normal in the ACC of CTCF HT mice (WT, n = 7; HT, n = 6; average of fEPSP slopes for the last 4 min; WT, 132.1 ± 3.3%; HT, 135.1 ± 5.0%; unpaired *t*-test; $p = 0.6123$).

(C, D) L-LTP was also normal in the ACC of CTCF HT mice (WT, n = 6; HT, n = 4; average of fEPSP slopes for the last 8 min; WT, 164.2 ± 10.1%; HT, 159.8 ± 9.7%; unpaired *t*-test; $p = 0.7700$).

(E) Cortical basal transmission was not attenuated in CTCF HT mice (WT, n = 10; cKO, n = 11; repeated measure two-way ANOVA, effect of genotype, $F_{1,19} = 0.2576$; $p = 0.6176$).

CTCF deficiency causes early signs of neurodegeneration in aged mice

In the previous chapter, the first goal was to confirm the protein reduction in the CTCF cKO mice. For the accuracy of the experiments, it was important to clarify the age, at which the protein level is sufficiently reduced. Although the *CaMKII-Cre* mouse line starts to express Cre around 4 weeks of age, I was only able to detect a sufficient level of protein reduction around 12 weeks of age due to CTCF's slow turnover rate. Therefore, in chapter II, I used 12-15-week-old mice. As Hirayama et al. (Hirayama et al., 2012) had previously shown that CTCF controls neural development during the postnatal period, I questioned age-specific roles of CTCF. I have found that CTCF cKO and HT mice with CTCF deletion in the early adulthood exhibit an impairment in remote memory, but roles of CTCF in the late adulthood has not been speculated.

To assess the role of CTCF in the late adulthood, I used the immunohistochemistry (IHC) technique and stained 30-week-old CTCF cKO mice's brain with neuronal nuclear antigen (NeuN) and glial fibrillary acidic protein (GFAP). NeuN is a neuronal specific nuclear protein that is commonly used as a biomarker for neurons (Rodney, 1999). GFAP is an intermediate filament protein mainly expressed by astrocytes (Hol and Pekny, 2015). It is widely used as a biochemical marker for gliosis and glioma. In this study, I used GFAP signal to detect reactive gliosis, which is a heterogeneous reactive response of glial cells in response to an injury in the central nervous system

(CNS) tissue (Burda and Sofroniew, 2014). When I stained hippocampus of the 30-week old CTCF cKO mice with DAPI and CTCF, I was able to confirm that CTCF expression is sufficiently decreased in the CA1 pyramidal cell layer (Figure 17). However, I also observed a decrease in the DAPI signal, indicating that the number of pyramidal cells has been decreased. Moreover, I found an increase of DAPI signal outside the CA1 cell layer, suggesting a possibility of an increase of non-neuronal cells. This led me to quantitatively assess the size of the cell layer by staining hippocampus with NeuN and measuring the width of the CA1 layer. As a result, I found that the width of CA1 layer is significantly smaller in the CTCF cKO mice, which implies cell death of the CTCF deficient excitatory neurons (Figure 18). Then, to characterize the increased DAPI signal around the CA1 cell layer, I stained the hippocampus and ACC of the aged CTCF cKO mice with DAPI and GFAP. Interestingly, I found a robust increase of GFAP signal in both regions, which is a sign of reactive gliosis (Figure 19). Together, the results show that long-term loss of CTCF causes cell death with reactive gliosis in response. These signs of neurodegeneration suggest a possible role of CTCF as an anti-degeneration factor in the adult brain. To validate that cell death had not occurred at earlier age, I stained the 20-week-old mouse brain with DAPI, NeuN, and GFAP. I found an increase of reactive gliosis in the CTCF cKO mice but did not find any signs of cell death (Figure 20). This result confirms that memory impairment phenotypes I observed in the CTCF cKO mice previously were not due to cell death.

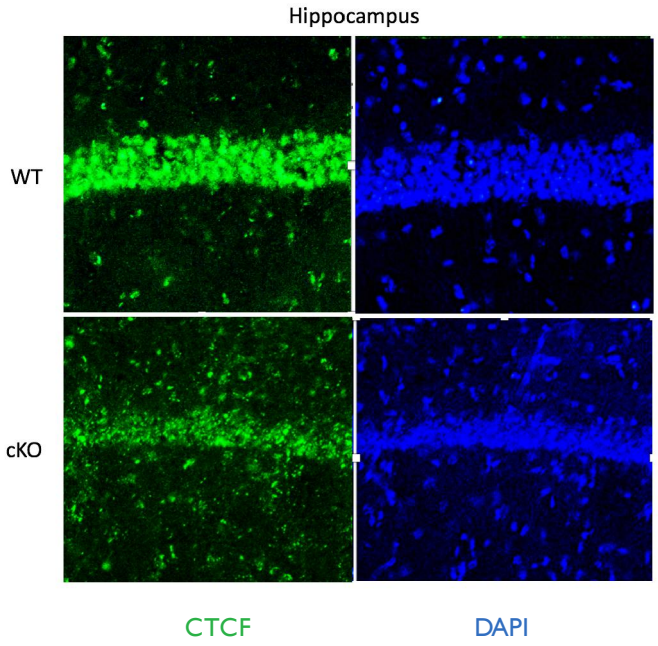

Figure 17. 30-week old CTCF cKO mice show decreased CTCF and DAPI signal in the CA1 layer

Immunohistochemistry analysis showed that CTCF cKO mice have reduced CTCF and DAPI signals in the hippocampal CA1 region (Blue: DAPI, Green: CTCF).

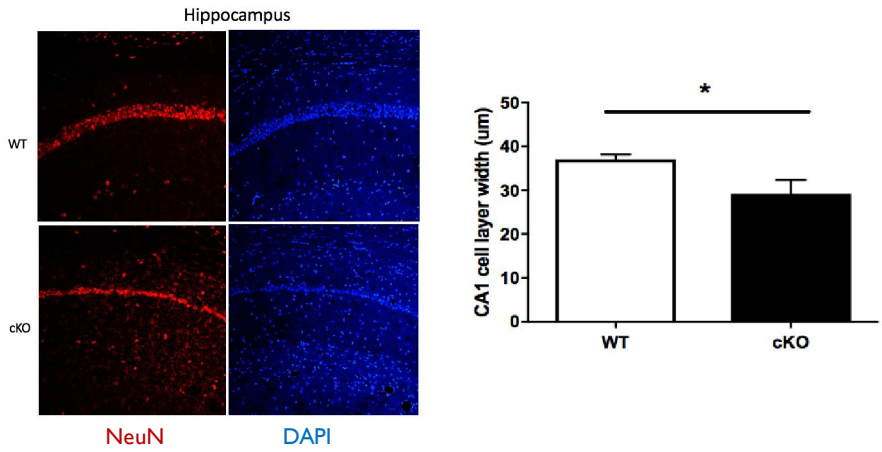

Figure 18. The width of CA1 layer is decreased in the 30-week-old CTCF cKO mice

NeuN-stained CA1 layer width was reduced in CTCF cKO mice (Blue: DAPI, Red: NeuN; WT, n=6; cKO, n=5; WT, 37.1 ± 1.047%; cKO, 29.13 ± 3.171%; unpaired *t*-test; $p = 0.0296$).

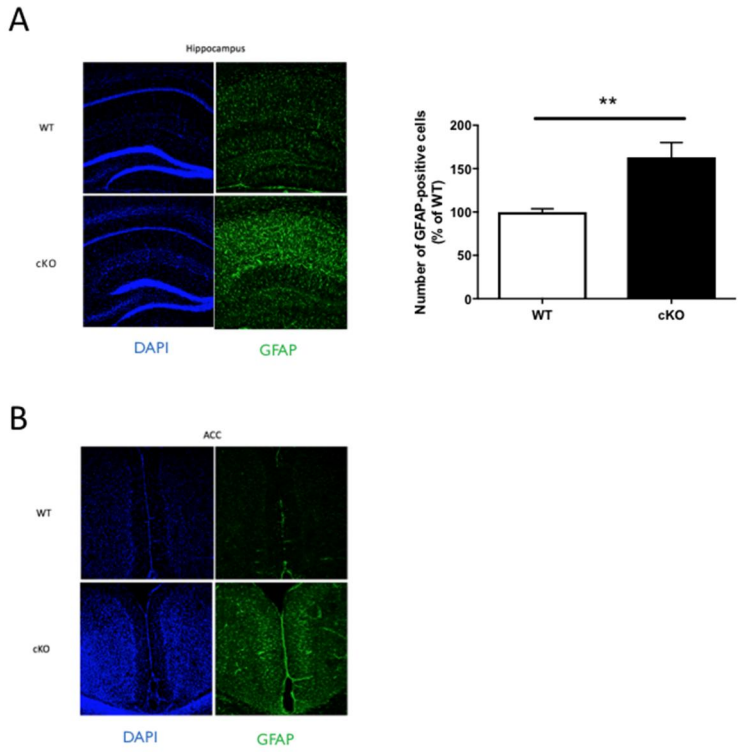

Figure 19. 30-week-old CTCF cKO mice have increased GFAP signal

(A) The number of GFAP-positive cells was significantly higher in the hippocampus of CTCF cKO mice (Blue: DAPI, Green: GFAP; WT, n=5; cKO, n=4; WT, 100 ± 3.738%; cKO, 163 ± 17.13%; unpaired *t*-test; $p = 0.005$).

(B) The GFAP signal was also higher in the ACC of CTCF cKO mice (Blue: DAPI, Green: GFAP).

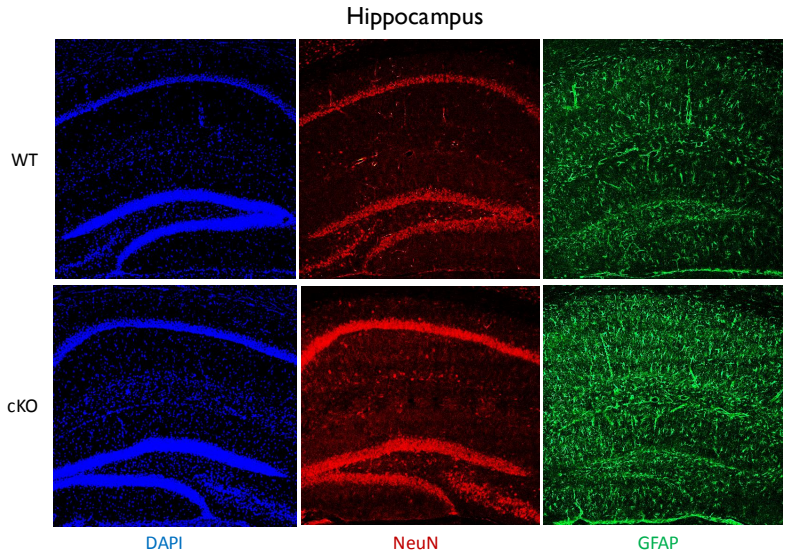

Figure 20. 20-week-old CTCF cKO mice have increased GFAP signal but show no signs of cell death (Blue: DAPI, Red: NeuN, Green: GFAP).

DISCUSSION

CTCF is a versatile protein with chromatin remodeling and transcriptional regulation activities. CTCF and its binding sites are known to be well-conserved across species. In this chapter, I examined the CTCF HT mice and found that they exhibit a partial impairment of remote spatial memory. This result revealed that CTCF also participates in the regulation of remote memory in inhibitory neurons. In the second half of the chapter, I assessed the consequences of long-term loss of CTCF and found an increase of cell death and reactive gliosis in the aged CTCF cKO mice. This suggested that CTCF deletion leads to early appearance of neurodegeneration.

I found that CTCF in inhibitory neurons also help regulate remote memory, and it is interesting how CTCF plays a similar function in two different types of neuron. In hippocampus and ACC, the two regions I examined in this study, the number of excitatory neurons is much higher than that of inhibitory neurons. Also, the excitatory and inhibitory neurons have very different functions. Excitatory neurons release neurotransmitters, such as Acetylcholine (Ach), that bind to receptors and depolarize the postsynaptic membrane. Inhibitory neurons, on the other hand, release a different type of neurotransmitters, such as Gamma Aminobutyric Acid (GABA), that bind to receptors and hyperpolarize the postsynaptic membrane (Bittner et al., 2017). Simply put, excitatory neurons increase the likelihood of the action potential firing, while inhibitory neurons decrease it. Inhibitory neurons support the

excitatory neurons that actually carry out a molecular function. Because the two types of neurons have different functions, it seems logical to expect that CTCF differentially regulates the two neurons to regulate remote memory. One hypothesis for the result of this study is that CTCF deletion impairs the inhibitory function by decreasing GABA concentration or changing the number of inhibitory synapses. This may disable inhibitory neurons from inhibiting the excitatory cells, leading to over-excitation. Abnormally high excitability is implicated in many brain disorders (Nelson and Valakh, 2015), and it may have contributed to impairment of remote memory. Another hypothesis is that CTCF deletion over-activates inhibitory cells, which leads to under-activation of the excitatory cells. Inhibitory neurons are known to be involved in neuronal oscillations, neurogenesis, and excitation balance in adult mammalian brain (Gonzalez-Burgos and Lewis, 2008). It is likely that CTCF deletion undermines the excitatory/inhibitory balance or functional homeostasis that disrupts systems consolidation. It will be interesting for future studies to find out to which direction the excitatory/inhibitory balance has been shifted. Miniature EPSC/IPSC recording and spontaneous action potential experiment using the patch recording will be able to show the homeostasis changes at a cellular level. Also, it will be fascinating to perform these experiments both in hippocampus and ACC to compare the region-specific effects, as CTCF deficient mice exhibited cortex-specific impairment of memory. Lastly, performing RNA-seq of pooled CTCF deficient inhibitory cells will help reveal the inhibitory cell-specific gene expression changes in comparison to the previous cortical neuron RNA-seq data.

In the second part of this chapter, I showed that aged CTCF cKO mice show signs of neurodegeneration. For the consistency of the story, I only examined some of the evident neurodegeneration phenotypes, but it will be meaningful to further assess the age-dependent roles of CTCF in detail. Previously, Hirayama et al. (Hirayama et al., 2012) showed that CTCF is critical for neural development, and this study has shown that CTCF regulates systems consolidation process during adulthood. However, CTCF's role in aged brain is still unclear. Although CTCF cKO mice showed strong signs of neurodegeneration, they were viable at least up to 40 weeks of age, as far as I observed. Therefore, this mouse line will be a useful model for studying neurodegeneration-related phenotypes in vivo. Investigation on how CTCF regulates gene expression and cellular properties in the late adulthood will provide an insight on CTCF's age-dependent differential roles.

CHAPTER IV

CONCLUSION

CONCLUSION

In this thesis, I have investigated the roles of CTCF in mature neuron and how CTCF is required for systems consolidation. I have assessed CTCF's role in two different types of neuron and have also shown how long-term CTCF deletion leads to early neurodegeneration.

In Chapter II, I focused on CTCF's role in excitatory neuron. I generated CTCF conditional knockout (cKO) mice by crossing the floxed CTCF line with *CaMKIIa-Cre* line, which expresses Cre recombinase in the forebrain excitatory neurons starting from 4–5 weeks of age. This enabled me to circumvent the lethal effect of postnatal CTCF deletion, and the CTCF cKO were viable at least until ~8 months of age with no obvious health abnormalities. I trained CTCF cKO mice in several memory tasks and tested them at two different time points: 1 day or 4 weeks after training. I found that CTCF cKO mice specifically exhibit impairments in the remote memory test. Then, I performed electrophysiological experiments to verify the underlying mechanisms at the synaptic level. Through hippocampal and ACC slice field recordings, I found that cortical synaptic plasticity and basal transmission impairments contribute to the remote memory deficit. Lastly, to define molecular mechanisms underlying these phenotypes, I conducted RNA-seq of CTCF KD cortical neuron cultures and analyzed the data using several bioinformatics tools. As a result, I found that CTCF deficient cells have altered expression of genes that are involved in functions like cell adhesion,

memory, and synaptic plasticity. Also, IPA analysis revealed that remote memory, LTP, and synaptic transmission are predicted to be downregulated in the CTCF deficient group, supporting the phenotypes of the CTCF cKO.

In Chapter III, I generated CTCF HT mice to examine CTCF's role in inhibitory neurons. I discovered that deletion of CTCF in inhibitory neurons also results in a partial impairment of remote memory. These results suggested that CTCF also plays an important role in inhibitory neurons and that CTCF deletion changes the function of interneurons, which possibly leads to an abnormal activity excitatory cells, causing a remote memory impairment. Also, I examined the brain of aged CTCF cKO mice and found that long-term loss of CTCF causes inflammatory responses and cell death. These results suggest that appropriate expression of CTCF is necessary for cell survival and neurogenesis.

Many previous studies have focused on the role of chromatin remodeling complexes during development (Hota and Bruneau, 2016; Inayoshi et al., 2006). To my knowledge, this is the first study to reveal CTCF's function in excitatory and inhibitory neurons of the mammalian adult brain. Also, I have found a novel association between CTCF-mediated chromatin remodeling, transcription, and memory consolidation. Future studies may investigate the difference in the mechanisms of recent and remote memory, using the CTCF deficient mice. Also, human genome is known have many CTCF binding sites (Kim et al., 2007), and it has been reported that several de novo mutations of CTCF in individuals cause intellectual disability (Gregor et al., 2013). Therefore, further investigations on the DEGs from the

RNA-seq data may provide more information on how CTCF-mediated chromatin architecture and gene regulation support memories to last.

Recently, Sams et al. (Sams et al., 2016) reported on the role of CTCF in hippocampus-dependent memory. They created CTCF cKO mice using a similar strategy of crossing the floxed CTCF mice with *CaMKIIa-Cre* line to inhibit the CTCF expression in post-mitotic excitatory neurons. However, their results were partially and yet significantly different from our present results. These discrepancies may be due to several factors, and we feel that it is noteworthy to go over the differences. While Sams et al. (Sams et al., 2016) similarly reported memory-related deficits in the CTCF cKO mice, they showed that the Cre-dependent CTCF deletion started at 1 week of age and a significant decrease of the protein level was reached by 8 weeks of age. However, our *CaMKIIa-Cre* line started its Cre expression at 4 weeks of age as previously reported (Liu et al., 2010; Tsien et al., 1996), and a sufficient level of protein reduction was only reached at 12 weeks of age (Figure 1C). Therefore, for most of the experiments, Sams et al. (Sams et al., 2016) used 10- to 12-week-old CTCF cKO mice, while we used 12- to 15-week old mice, which had avoided developmental effects of the gene deletion. Furthermore, Sams et al. (Sams et al., 2016) reported that their CTCF cKO mice died after 17 weeks of age with weight loss, while our CTCF cKO mice were viable for more than 40 weeks of age and exhibited no apparent health abnormalities. In addition to physical phenotypes, the memory-related deficits also appeared to be different between the two studies. The CTCF cKO mice in Sams et al. (Sams et al., 2016) exhibited impaired hippocampal LTP with disrupted recent

memory in the cued fear conditioning and MWM tests. However, our CTCF cKO mice exhibited normal hippocampus-dependent phenotypes with dramatically impaired cortex-dependent remote memory. Moreover, while the RNA-seq data showed a similar result in *Pcdh* expression change, Sams et al. (Sams et al., 2016) showed a higher number of upregulated genes, while our present study had a higher number of upregulated genes in the DEG list. In summary, Sams et al. (Sams et al., 2016) focused on investigating the effect of CTCF deletion early in life and its roles in the hippocampus, while we focused on the effect of CTCF deletion in adulthood and its roles in the cortex-dependent remote memory. The differences in the results also may arise from the difference in the mouse lineage background, as it has been previously reported that recombination patterns and according phenotypes of the same Cre mouse line may differ due to the genetic background and breeding strategies (Fex et al., 2007; Gil-Sanz et al., 2015). Also, for the RNA-seq experiment, Sams et al. (Sams et al., 2016) used hippocampal tissue from 10-week-old mice, while we used CTCF KD cortical cultures, which can also account for the difference in the data. The early lethality observed in the previous study (Sams et al., 2016) may be due to the apoptosis of pyramidal cells, which was not detected in our mice. Therefore, further investigations are needed to find the exact mechanisms underlying the differences between the two studies. Also, it may be interesting to explore the age-dependent role of CTCF in neurons, since the timing of the protein deletion accounts for the main difference between the two CTCF cKO mouse lines. It may be plausible to think that CTCF changes its main regulatory function in the brain with the

age.

The findings of this study provide a groundwork for investigations on the role of 3D genome organization during systems consolidation. Here, I propose a novel mechanism, through which CTCF alters chromatin structure to regulate remote memory. However, this study falls short of data showing how chromatin structure is actually changed during remote memory formation and maintenance. Therefore, future investigations are necessary to establish and certify the novel mechanism of CTCF's remote memory regulation through chromatin remodeling. One idea for future studies is to examine the geometrical changes of chromatin during the systems consolidation process in a time-dependent manner. Advanced chromosome conformation capture techniques, such as 4C and Hi-C, may be useful because they allow comprehensive and unbiased analysis of chromatin and protein interactions (van de Werken et al., 2012). It may also be interesting to examine gene expressional changes at specific time points during the systems consolidation process. This way, future studies can show what kind of gene expression and molecular changes are occurring during the 30-day remote memory formation process.

As learning and memory are core parts of human cognition, understanding the mechanisms of memory can help people comprehend who they are better. As the percentage of people suffering from neurological disorders has been increasing, economic pressure and therapeutic needs have also been keep rising (Thakur et al., 2016). I expect that CTCF deficient mice used in this thesis can be further used as a remote memory mouse model in

future investigations. Also, the DEG list from the RNA-seq data may be used as a database for investigating specific gene's contribution to remote memory. For example, IPA analysis showed several attention-grabbing genes, such as dopamine receptor 1 (*Drd1*), gamma-aminobutyric acid type A receptor alpha 5 (*Gabra5*), *Fos*, and *Arc*. As membrane receptors are known to regulate synaptic response and cell signaling, while immediate early genes regulate transcription and synaptic plasticity, further investigations may reveal a novel function of a gene in relation to remote memory. Since there is no report on the functional relationship between *Pcdh* genes and remote memory, it will be interesting to find how Pcdhs alter chromatin formation and participate in the remote memory formation. Detailed analysis of DEGs will also reveal more detailed downstream mechanisms of CTCF regulation of remote memory. Also, investigation of memory engram dynamics over time in different brain regions can help reveal what molecular changes are occurring in the memory-bearing cells. Using powerful techniques such as calcium imaging, future studies can uncover cellular dynamics and regional activities underlying remote memory consolidation.

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국문초록

기억의 분자적 메커니즘은 해마가 관여하는 단기기억에 대해 주로 연구되어왔다. 몇일 단위가 아닌 몇달 단위로 지속되는 장기기억은 해마가 아닌 대뇌피질에서 담당하는 것으로 알려져 있는데, 이에 대한 분자적 메커니즘은 아직 많이 연구되어 있지 않다. 장기기억에는 다양한 후성 유전 메커니즘이 중요한 역할을 한다는 보고들이 있지만, 염색질 구조의 입체적 변화와 관련된 단백질들이 시냅스 가소성과 기억의 응고화에 어떻게 기여하는지는 자세히 알려져 있지 않다. CCCTC-binding factor (CTCF)는 7개의 징크 핑거를 가진 단백질로 전사 인자로써의 역할이 잘 알려져 있으며, 염색질 구조 변화에도 관여한다는 것이 이미 알려져 있다. 염색질의 3차원적 구조 변화에 따른 기억의 변화에 대해 알아보기 위해 CTCF 단백질의 발현이 저하된 생쥐를 사용하여 다양한 실험을 진행 하였다. CTCF의 발현이 흥분성 뉴런에서 억제된 생쥐 (CTCF cKO)는 공포 조건화 실험과 모리스 수중 미로 실험에서 정상적인 단기 기억력을 보였지만, 같은 실험을 약 4주 뒤에 진행하였을 때는 심각하게 손상된 장기기억력을 보였다. 뿐만 아니라, 전기 생리학 실험을 통해 CTCF cKO 생쥐들은 해마에서 정상적인 시냅스 가소성을 보이지만 전방대상피질에서는 시냅스 가소성과 신경 전달이 저하된 것을 확인하였다. 이러한 결과에 대한

분자적 근거를 찾기 위해 바이러스를 이용해 뉴런 컬쳐에서 CTCF를 knockdown (KD) 시킨 뒤 RNA 염기서열 분석 (RNA-sequencing)을 진행하였다. 그 결과, CTCF KD 그룹에서 약 400개개에 달하는 유전자에서 발현 정도가 변한 것을 보았다. 또한, gene ontology (GO) enrichment assay를 통해 세포 부착, 시냅스 가소성, 기억 등에 관련하는 유전자들의 발현이 변한 것을 관찰할 수 있었다. CTCF의 역할에 대해 더 자세히 알아보기 위해 CTCF의 발현을 억제성 뉴런에서만 저하시켰다. 그 결과, 생쥐가 부분적으로 손상된 장기 기억을 가지고 있다는 것을 발견하였고, 이는 CTCF 단백질이 흥분성과 억제성 뉴런에서 비슷하게 장기 기억을 조절하는 역할을 한다는 것을 보여주었다. 또한, CTCF의 발현이 장기간 억제되었을 때 어떠한 결과가 나타나는지 보기 위해, 약 30주령의 나이든 생쥐의 뇌를 관찰 한 결과 해마와 전방대상피질에서 세포 사멸과 신경아교증증이 증가한 것을 보았다. 이러한 결과들을 통해, CTCF 단백질이 전사 조절과 염색질 구조 변화를 통해서 대뇌피질에 저장되는 장기 기억을 조절한다는 것을 처음으로 발견하였고, CTCF의 발현이 장기간 저하되었을 때 신경 퇴행이 일어난다는 것을 보여주었다.

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주요어 : CTCF, 전사, 염색질 리모델링, 기억의 응고화, 신경 퇴행

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